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## RESEARCH ON OXYGEN TOXICITY AT THE CELLULAR LEVEL

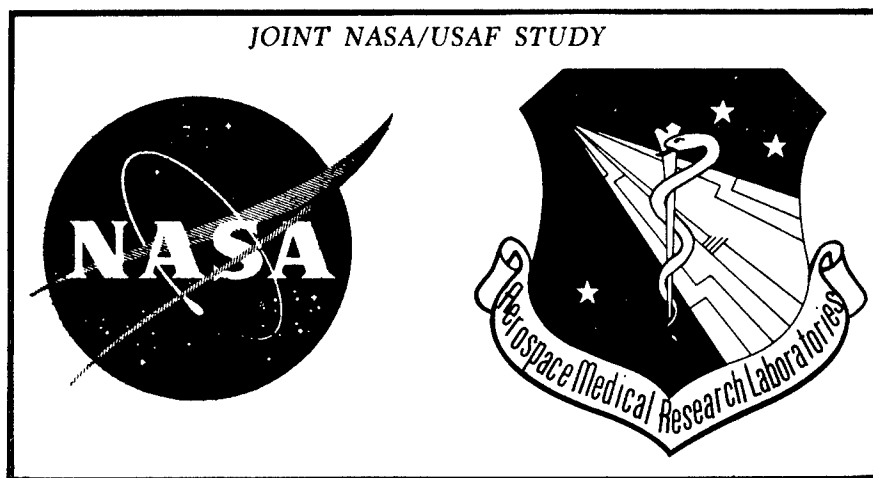
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## FOREWORD

This research was initiated by the Toxic Hazards Division, Biomedical Laboratory, Aerospace Medical Research Laboratories, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio 45433. The research was conducted by IIT Research Institute, Technology Center, Chicago, Illinois, under Contract No. AF 33(615)-2164, at the facilities of the Altitude Protection Branch, Life Support Division, and the Toxic Hazards Division, Biomedical Laboratory, Wright-Patterson Air Force Base. This work was sponsored by the National Aeronautics and Space Administration under NASA Defense Purchase Request No. R-87. The research began 15 April 1965 and was completed 15 June 1966.

The program was monitored at AMRL by Harold P. Kaplan, Captain, USAF, MC, and initially by Philip Felig, Captain, USAF, MC; Farrel R. Robinson, Major, USAF, VC; and David T. Harper, Jr., Captain, USAF, MC. Contributing personnel at AMRL included Joseph Young, Master Sergeant, and Jack L. Gillmore, Airman First Class, under the direction of Dr. Kaplan. We wish to acknowledge the continued guidance and counsel contributed by Dr. Kaplan.

IITRI personnel on location at AMRL were Mr. James Q. Kissane, Mr. Raymond W. Bieber, and Dr. Willis H. Riesen. Contributing personnel at IITRI included Mr. M. Kardatzke, who participated in the original experimental design and the final statistical treatment of the data, and Dr. R. Ehrlich, Director, Life Sciences Research, and Dr. E. J. Hawrylewicz, Assistant Director, who provided administrative guidance to the program.

The final report has been identified as Report No. IITRI-L6027-13.

This technical report has been reviewed and is approved.

WAYNE H. McCANDLESS  
Technical Director  
Biomedical Laboratory  
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## ABSTRACT

The use of pure oxygen atmospheres at reduced pressure in manned spacecraft has stimulated new research interest. Exposure of humans to pure oxygen at total pressures of 5 to 7 psia for up to 30 days has generally produced only subjective and occasional symptomatic distress. Nevertheless, studies with animals have clearly documented toxicity if the exposure to pure oxygen is at higher total pressures. The selection of suitable oxygen atmospheres for manned space flight and the development of protective measures against toxic manifestations will be considerably facilitated by a clearer understanding of the toxic syndrome. Rats, dogs, and monkeys were exposed to pure (98.5%) oxygen at 1 atm and 1/3 atm in a closed, recirculating chamber and in a non-recirculating chamber. Rats exposed at a pressure of 1 atm showed significant reduction in efficiency of liver mitochondrial oxidative phosphorylation. Weight loss accompanied this change. Insignificant changes were found in liver or lung mitochondrial oxygen consumption; liver or lung NAD, NADH, or NAD/NADH; and arterial blood lactate and pyruvate, and lactate/pyruvate ratio. A composite index of oxygen toxicity was derived from a multivariant data analysis. Exposure of rats to pure oxygen at 1/3 atm showed slight elevation in P/O ratio in 7 days. The ratio returned to normal in 2 weeks and remained so for 8 months. No significant weight changes were observed. Only preliminary experiments with the dogs and monkeys were conducted, therefore no conclusions can as yet be made. There is an indication that monkeys are more resistant to oxygen toxicity at 1 atm than rats.

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## SECTION I

### INTRODUCTION

The use of pure oxygen atmospheres at reduced pressure in manned spacecraft has stimulated new research interest in the area of oxygen toxicity. Exposure of humans to pure oxygen at total pressures of 5 to 7 psia for periods up to 30 days has generally produced only subjective and occasional symptomatic distress (ref. 1,2). Nevertheless, studies with animals have clearly documented toxicity if the exposure to pure oxygen is at higher total pressures. Obviously, the selection of suitable oxygen atmospheres for manned space flight and the development of protective measures against any toxic manifestations will be considerably facilitated by a clearer understanding of the toxic syndrome.

Previous morphological and metabolic studies at the Aerospace Medical Research Laboratories\* indicated (ref. 3) that toxicity in animals exposed to pure oxygen at 1 atm was manifested by changes in mitochondrial structure. Furthermore, the finding by Felig and associates (ref. 4) that lactate protects against oxygen toxicity suggested involvement of the oxidation-reduction state of the pyridine nucleotides in the toxic mechanism. These findings and the known oxygen sensitivity of respiratory enzymes demonstrated by many investigators in vitro (ref. 5) more than suggest that the ultimate mechanisms might be sought at a sub-cellular level.

This investigation was concerned with the metabolic processes that result from exposure of experimental animals to pure oxygen environments at various pressures. Tissue were examined at a subcellular (i.e., mitochondrial) level for changes in respiratory indexes and activities of selected enzyme systems.

The prime objective was the investigation of effects of exposure of animals to the atmospheric conditions used in manned space flight. The experiments were of necessity carried out under conditions that simulate the manned space cabin. Thus ground-based environmental chambers of two different types were used. Three different species of experimental animals, including primates, were exposed to the oxygen atmospheres.

The oxygen pressure presently used in the manned space cabin is  $1/3$  atm. Since there is virtually no information available concerning the effects of exposure to oxygen at  $1/3$  atm upon cellular respiration, studies at 1 atm were also carried out. We realized that any acute toxic manifestations observed at 1 atm would be quite different from the effects of oxygen at  $1/3$  atm. Significantly, in previous studies at the 6570th AMRL

\*Hereafter referred to as 6570th AMRL.

some of the morphological changes observed within 1 week at 1/3 atm were similar to those observed within 1 or 2 days at 1 atm. Acute changes observed at 1 atm merely suggested the types of changes that might be sought in animals exposed to oxygen at lower pressures.

In the experiments described, animals were subject to earth's gravitational force, a condition that differs from present cabins used in manned space flight. With respect to temperature and relative humidity, conditions are similar.

The theory underlying this approach is simply that by using the environmental conditions described with several animal species, we can increase the speed and probability of detecting any toxic manifestations of oxygen exposure. Obviously, the biochemical mechanisms of oxygen toxicity cannot be studied unless conditions are such that a toxic syndrome is produced. The wide variation in susceptibility is seen in the case of the laboratory white rat. In previous studies at the 6570th AMRL, different strains have shown either complete tolerance or lethality within several days upon exposure to pure oxygen at 1 atm.

This report first describes the environmental chambers, experimental animals, and experimental runs. Then the experimental results are divided into those obtained in exposures to 1 atm and those to 1/3 atm of oxygen. Section IV discusses the interrelationships of the statistical and the biochemical findings, the effects of animal species and environmental parameters, the phenomena of adaptation and recovery, and the relation to other research findings. Only summary tabular and graphic data are presented in the main section of the report. Detailed analytical methods and replicate analyses are presented in the Appendix. A considerable portion of the data was subjected to a standard analysis of variance.

The data provided biochemical and physiological indications of the susceptibility of rats to oxygen toxicity at 1 atm. Furthermore, an early adaptive mechanism is suggested in the exposure of rats to oxygen at 1/3 atm. The data indicate that, as a result of exposure to pure oxygen, liver mitochondria are the site of significant changes in respiratory metabolism. By implication, the impairment is in the pathways of electron flow involved in energy utilization at the ultrastructural level within the tissue cell. Although more elaborate techniques may be required to fully understand the biochemical mechanisms, nevertheless the indexes that were found suggest that additional studies are required to further delineate the processes of adaptation in the oxygen environment, recovery in air, and protection by administration of specific substances. The protective substances will further suggest certain metabolic pathways that are probably impaired in oxygen toxicity.



## SECTION II

### EXPERIMENTAL METHODS AND PROCEDURES

#### A. Environmental Chambers

Animals were exposed to pure oxygen atmospheres in two different environmental chambers, the Felig-Lee chamber and Thomas domes.

##### 1. Felig-Lee Chamber

The Felig-Lee chamber is a closed system and includes an air lock for entry, facilities for maintaining the atmospheric composition, and sampling ports for gas analysis (ref. 4). A block diagram of this chamber is shown in Figure 1. The aluminum chamber has an external fabric insulation and houses 72 individual wire-mesh rat cages. The chamber is entered through an air lock that is first brought to the desired oxygen concentration to prevent nitrogen contamination. The chamber can accommodate two persons for surgical procedures. Oxygen concentration is maintained at  $98.5 \pm 1\%$ , relative humidity at 40 to 60%, and temperature at 74 to 76°F. In our experiments, the chamber was used only at 1 atm (760 mm Hg) pressure to ensure that all leaks would be outboard.

The chamber atmosphere is recirculated through a closed pump with aviator's oxygen for initial purge and subsequent replacement. The carbon dioxide concentrations are maintained below 0.2% by circulation through lithium hydroxide. Sampling for gas analysis is continuous; intermittent analysis on a Bendix time-of-flight mass spectrometer showed no detectable contaminants.

Since the chamber walls are visually opaque, morbidity and mortality are determined by inside observers twice daily. When deaths occur before the end of the exposure period, the animal carcasses are removed for autopsy.

During the initial experimental runs all gas flow was controlled by manual operators on a continuous day-and-night basis. Subsequently, the chamber was equipped for automatic control of oxygen pressure and gas flow; relative humidity and temperature remained under manual control.

After the animals were placed in the chamber, 1 hr was allowed for chamber atmospheric equilibration prior to initiation of the run.

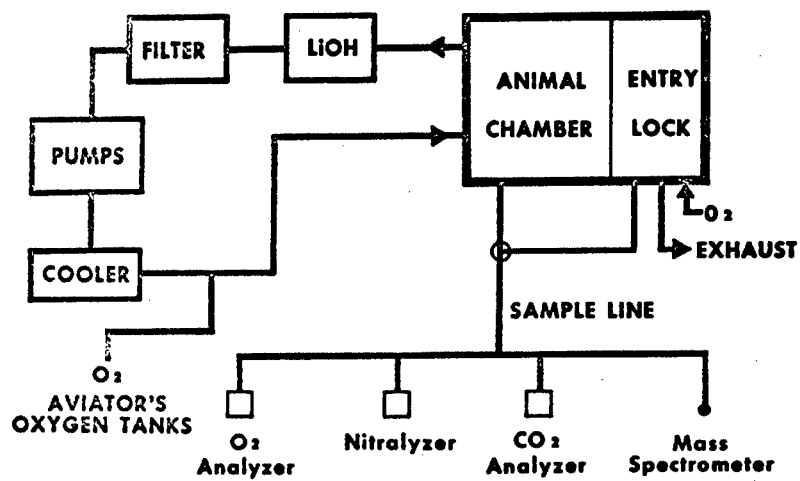


Figure 1. Block diagram of Felig-Lee chamber facilities (direction of flow of atmosphere is indicated by arrows).

## 2. Thomas Dome

The Thomas facilities were designed to perform inhalation studies with space cabin materials. The dome-shaped exposure chambers, 9 ft tall and 12 ft in diameter, provide visibility, evacuation to 1/3 atm, and uninterrupted exposure by a vertical airlock system (Figures 2 and 3). The tops of the domes can be separated for loading with animals. Automated operation requires care by only one person. Oxygen, carbon dioxide, oxygen flow rate, temperature, absolute pressure, and relative humidity are controlled in each dome independently.

Liquid oxygen tanks supply gaseous oxygen at 50-psi pressure and 72°F. Filtered air can be used to make up oxygen-nitrogen mixtures, although only pure oxygen was used in these studies. Equipment redundancy prevents experimental loss due to equipment breakdown.

Monkey cages hold 6 monkeys; dog pens hold a total of 10 to 12 animals.

Operators entering the chamber use walk-around oxygen bottles. A 60-min denitrogenation period is required to prevent bends. Upon entering the air lock, the operator connects to the permanent oxygen supply, purges to 98% oxygen, and then depressurizes to operating pressure. He then enters the dome via a ladder to care for the animals.

The outside observer maintains complete visual and audio contact with the technician inside the air lock or the dome.

This facility has highly sophisticated equipment capable of simulating the atmospheric environment of space cabins. The use of a variety of animals in the Thomas domes with oxygen-rich environments at low pressure constituted a major phase of the experimental program.

### B. Experimental Animals

In order to study the variety of responses that mammals can show upon exposure to oxygen, several species of animals were used. A fuller understanding of these possibly diverse responses increases the probability of application to man. Since a large body of metabolic data is available for the albino laboratory rat, and because this animal has a high degree of genetic uniformity, the rat was the animal most widely used. Sufficient numbers of animals permitted statistical approaches to data analysis.

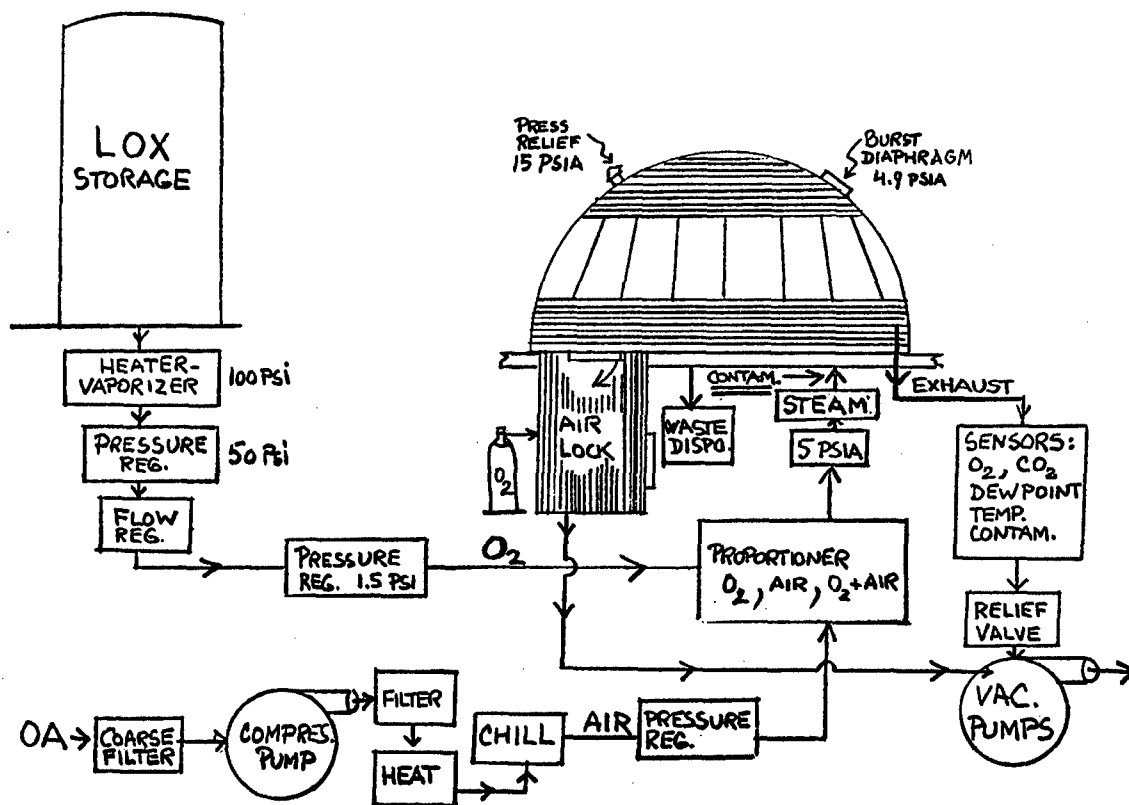


Figure 2. Flow diagram of Thomas dome (direction of flow of atmosphere is indicated by arrows).

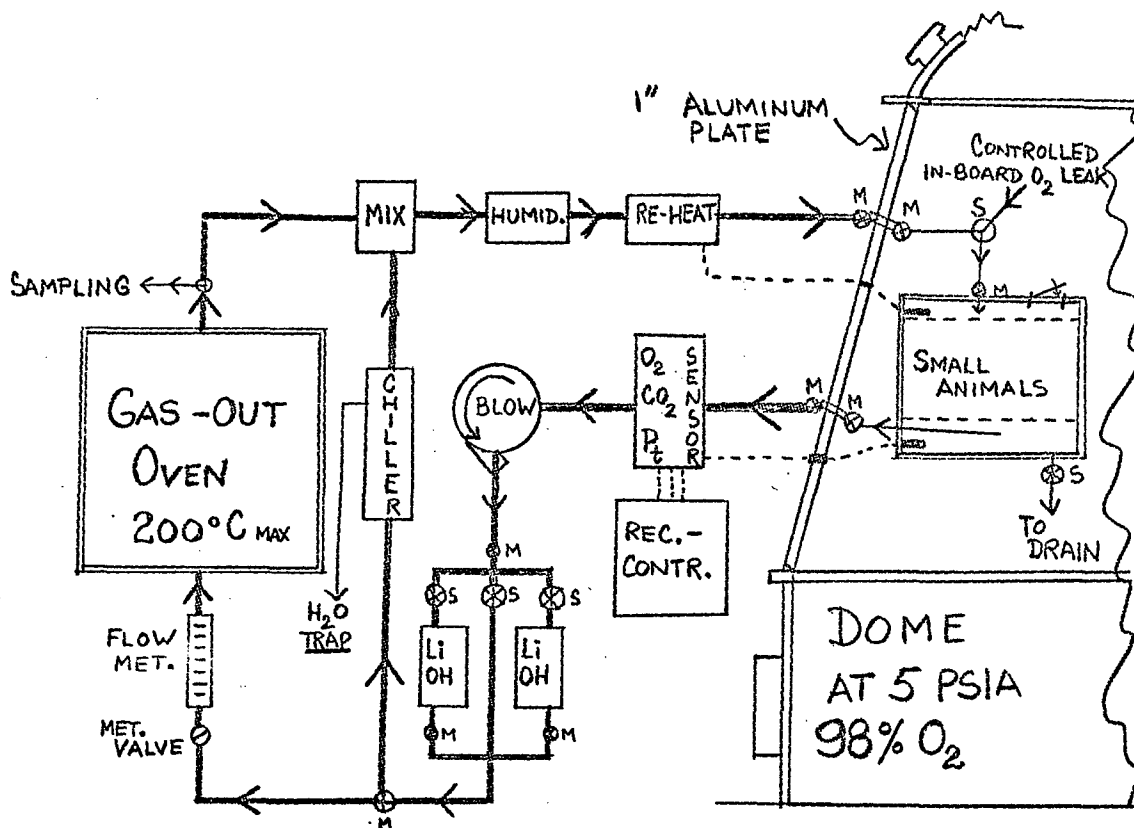


Figure 3. Flow diagram of Thomas dome as used with mixed atmospheres for small animals.

To study possibly divergent response patterns in larger animals, the dog and the monkey were chosen. The inclusion of a primate further increases the applicability of the data to the problems encountered in human exposure.

### 1. Rats

Male Sprague-Dawley rats weighing between 150 to 250 g were used for all control and oxygen exposure experiments. The choice of male animals was made to preclude biochemical effects that might arise during female menstrual cycles. The rats used in all biochemical experiments were furnished by one supplier (Harlan) with the exception of a long-term exposure in which animals were from another supplier (Carworth).

All rats were quarantined for periods of 3 to 7 days in the 6570th AMRL Vivarium prior to any experiments to determine their state of health. During this time the animals were observed for normal growth and normal pulmonary ventilation, i.e., the lack of "wheezers." Animals that appeared abnormal in this respect were not used.

### 2. Monkeys

Rhesus monkeys (*Macaca mulata*) weighing between 2.0 to 2.5 kg were selected as the primate of choice for determining the effects of oxygen exposures. Both male and female monkeys were used.

### 3. Dogs

The Beagle (*Canine domesticus*) was chosen as an additional species of higher order to determine effects of oxygen exposure. Both male and female dogs, weighing between 6.8 and 7.7 kg, were used.

## C. Atmospheres and Times of Exposure

Of prime importance in carrying out the experimental program was the selection of the oxygen pressures that would be investigated and the time periods of exposure that would reflect changes in tissue metabolism. The major limitation was the time period of lethality at each pressure. At pressures near the partial pressure of oxygen in the natural atmosphere (159 mm Hg), the period of exposure can be relatively long, i.e., a period during which probably only reversible changes occur.

In order to exaggerate the toxic effects of exposure to oxygen, higher pressures than those that supply the oxygen partial pressure in 1 atm of air were used. In U.S. space flights, astronauts breathe pure oxygen at 1/3 atm of pressure, which supplies one and two-third times the level of oxygen in 1 atm of air. At higher pressures, the toxic effects of oxygen will be increased with a corresponding decrease in the time to death.

Pressures of oxygen below 1 atm can produce physiological effects other than oxygen toxicity, namely, those caused by hypobaric pressures. These effects can include decreased solubility of gases in the blood, different rate of exchange in the alveoli, and stresses during adaptation to reduced pressure at the beginning and atmospheric air pressure at the end of the experiment.

The pressures of oxygen and the periods of exposure were chosen on the basis of previous morphological findings at 6570th AMRL. Rats exposed to pure oxygen at 1 atm for 24 to 48 hr showed hepatic structural changes that were similar to those exposed at 1/3 atm for 7 days. This apparently parallel morphology served as a basis for selection of the range of exposure periods for each oxygen pressure in our biochemical studies.

The limitation at 1 atm is almost 100% mortality in certain strains of rats (including the strain used in these experiments) exposed for periods longer than 3 or 4 days. No adaptation during exposure is then expected, although recovery in air after sublethal and reversible exposure to oxygen was investigated.

At 1/3 atm of pure oxygen the major considerations are the detection of any alterations in metabolism by the most sensitive biochemical indexes available. At this pressure, adaptation during exposure is observed. In all adaptation experiments the important consideration is to allow enough time after detection of initial damage so that full adaptation is reached. Our guideline was the allowance of at least double the time after detection of oxygen toxicity.

The final consideration in the selection of periods of exposure was that the speed of response in larger animals tends to be slower than in smaller ones. Hence periods of exposure for dogs and monkeys were generally longer than for rats, particularly at 1 atm.

#### D. Experimental Runs

Data from the experimental runs - including oxygen pressure, test animals, chambers, exposure intervals, and the types of biochemical analyses - are summarized in Tables I through IV.

The exposure intervals used at each pressure of oxygen are shown in Table III. The exposure periods common to both oxygen pressures are 24 hr at 760 mm Hg pressure and 168 hr (7 days) at 258 mm Hg pressure.

##### 1. Normal Animals

To develop proficiency with each of the biochemical analyses, rats were first analyzed without previous exposure to oxygen (Run N-1). These animals breathed air outside the chamber but were otherwise of the same species and weight range used later for oxygen exposure. The prime objective was to compile values sufficient for establishing the normal variance between animals and to check duplicate values with the same animal.

##### 2. Control Animals

During oxygen exposure in the chamber, animals drawn randomly from the same initial lot were exposed to air outside the chamber. These were designated control. Except when otherwise designated, separate sets of control animals were held to the end of each oxygen exposure period for simultaneous analysis with the experimental animals. These control animals are directly comparable to the corresponding experimental animals, rather than the "normal" animals just described.

##### 3. Recovery Experiments

In several runs some animals were removed from the chamber after the final exposure period to test recovery in air. Recovery should not be confused with adaptation, which can be observed during prolonged oxygen exposure.

#### E. Tissue Preparation and Biochemical Analyses

Biochemical analyses for indication of oxygen toxicity were chosen to reflect fundamental changes in intracellular metabolism and energy utilization. The tissues and biochemical analyses are shown in Table IV.



Table I. Summary of data obtained in experimental runs

Run No.	Oxygen Pressure, mm Hg	Animal	Chamber	Maximal Exposure Interval	Biochemical Analysis <sup>d</sup>
N-1 <sup>a</sup>		Rat	None		U, V, W
1, 2 <sup>c</sup>	760	Rat	Felig-Lee	72 hr	U, V, W
3, 4	750	Rat	Thomas	96 hr	U, V, W
5, 6	760	Rat	Felig-Lee	96 hr	U, V, W
7 <sup>c, b</sup>	750	Monkey	Thomas	170 hr	V
8 <sup>c, b</sup>	258	Monkey	Thomas	236 days	U, V, W
9 <sup>b</sup>	258	Dog	Thomas	236 days	U, V
10 <sup>c</sup>	258	Rat	Thomas	236 days	U, V
11 <sup>b</sup>	258	Monkey	Thomas	72 hr	U, V
12	258	Rat	Thomas	336 hr	U, V
13	258	Rat	Thomas	168 hr	U, V
14	750	Rat	Thomas	72 hr	U, V
15 <sup>b</sup>	760	Rat	Felig-Lee	96 hr	V
N-2 <sup>a, b</sup>		Rat	None		

<sup>a</sup>"Normal" animals in air at ground-level pressure.

<sup>b</sup>Modified procedures for tissue removal.

<sup>c</sup>Included an air recovery experiment.

<sup>d</sup>"U" designates liver mitochondrial Q (O/N), P/O, and LDH and lung mitochondrial Q (O/N) and LDH; "V" designates liver NAD/NADH and lung NAD/NADH; "W" designates arterial blood lactate/pyruvate.

Note: A detailed compilation is shown in Table A-I-1.

Table II. Environmental chambers, atmospheres, and experimental animals

<u>Chambers</u>	<u>Atmospheres</u>	<u>Animals</u>
Felig-Lee	98.5% oxygen at 760 mm Hg	Rats (Sprague Dawley, Harlan)
Thomas dome	98.5% oxygen at 750-760 mm Hg	Rats (Sprague Dawley, Harlan, and Carworth)
	98.5% oxygen at 258 mm Hg	Dogs (Beagle)
		Rhesus monkeys ( <u>Macaca mulata</u> )

Table III. Times of exposure

<u>Oxygen Pressure, mm Hg</u>	<u>Times of Exposure</u>						
750-760	6	24	48	72	96	hr	
258		24		72	168	336	hr 236 days

Table IV. Tissues and biochemical analyses

Animal Group	Tissue	Biochemical Analyses
U	Liver mitochondria	Q(O/N) and P/O ( $\alpha$ -ketoglutarate substrate) LDH total activity and isoenzymes
	Lung mitochondria	Q(O/N) (Succinate substrate) LDH total activity and isoenzymes
V	Whole liver	NAD, NADH, and NAD/NADH
	Whole lung	NAD, NADH, and NAD/NADH
W	Arterial blood	Lactate, pyruvate, and lactate/pyruvate

In earlier studies at 6570th AMRL the mitochondrion was shown to be morphologically sensitive to oxygen. The chief function of this organelle is to transform the energy inherent in anaerobic glycolytic products of cellular metabolism to a concentrated form readily tapped. The transformation involves the cyclic removal of hydrogen and carbon in aerobic metabolism. The hydrogen removed is transported with the aid of successive carriers ultimately to molecular oxygen. Ideally, the consumption of oxygen in this overall process is tightly coupled to the conversion of energy into adenosine triphosphate (ATP); in fact, the bulk of the energy inherent in glucose is made available during hydrogen and electron transport.

The efficiency of coupling of oxygen consumption to formation of high-energy phosphate bonds is indicated by the P/O ratio. The lactic dehydrogenase (LDH) activity reflects the enzymatic conversion of lactate to pyruvate, enabling the transition from anaerobic to aerobic metabolism. Specific organ LDH isoenzyme distributions have anaerobic/aerobic significance. In general, serum LDH activity shows systemic release of isoenzymes characteristic for a particular organ. Homogenate LDH patterns show this organ characteristic directly. In these studies, we measured mitochondrial LDH activities and isoenzyme patterns that may be significant to mitochondrial metabolism in particular.

The organs chosen for mitochondrial studies were liver and lung. Liver mitochondria were extensively investigated, enabling direct comparison with published data. Lung mitochondria were investigated because of the position of lung as the target organ in tissue confrontation by atmospheric oxygen.

Since a number of the aerobic citric acid cycle intermediates are coupled to pyridine nucleotides in their dehydrogenation, the redox ratios of these coenzymes are of considerable interest. These ratios reflect the direction of flow of hydrogen and electrons in their transport to molecular oxygen. In these studies, whole liver and lung NAD/NADH were investigated. The levels of the oxidized or reduced forms were considered individually as well as in the form of ratios. In view of the sensitivity of this system to tissue ischemia, special techniques of tissues removal were used.

To measure the redox state of the animal tissue generally, blood lactate and pyruvate were analyzed. The accumulation of lactate with reduction of pyruvate is used widely as a measure of predominant anaerobic metabolism with oxygen debt. However, a rise in pyruvate in itself indicates rapid glycolysis without the ability to form lactate, perhaps due to a deficiency of LDH. Hence both individual values and ratios were determined to gain the most overall picture of general tissue oxidative metabolism.

The following description of methods and analyses is presented in a summary form; a more detailed presentation is found in the Appendix.

### 1. Mitochondrial Respiration

Viable mitochondrial preparations were prepared from the livers of rat, dog, and monkey and from the lungs of rats by the methods of differential ultracentrifugation described by Schneider and modified by Lardy (ref. 6 and 7). Figure 4 shows the procedure used for fractionating the liver mitochondria.

The oxygen uptake was determined manometrically by the Warburg technique employed by Lardy and associates (ref. 7) using glucose-hexokinase trap for phosphate. Liver mitochondria respired and phosphorylated at an acceptable rate with alpha-ketoglutarate as substrate. However, lung mitochondria did not respire with this substrate, but did consume oxygen with succinate. Hence, while lung mitochondrial succinate  $Q(O/N)$  was determined, alpha-ketoglutarate  $Q(O/N)$  and  $P/O$  in subsequent data refer to liver mitochondria.

The volumes of oxygen consumed during respiration experiments were corrected to a milligram unit of mitochondrial nitrogen ( $Q(O/N)$ ). Protein analyses were performed by the photometric biuret method, the values of which were correlated to nitrogen analyzed for each tissue by the micro-Kjeldahl method.

Oxidative phosphorylation in liver mitochondria was determined by measuring the amount of inorganic phosphate converted to glucose-6-phosphate during the  $Q(O/N)$  measurement by way of the glucose-hexokinase-ATP trapping system. For this purpose, initial and final inorganic phosphate levels were analyzed according to the Fiske-Subbarow method and the differential phosphate calculated. The overall efficiency of oxidative phosphorylation is expressed as a ratio of the number of microatoms of phosphate converted to ATP to that of oxygen consumed ( $P/O$  ratio).

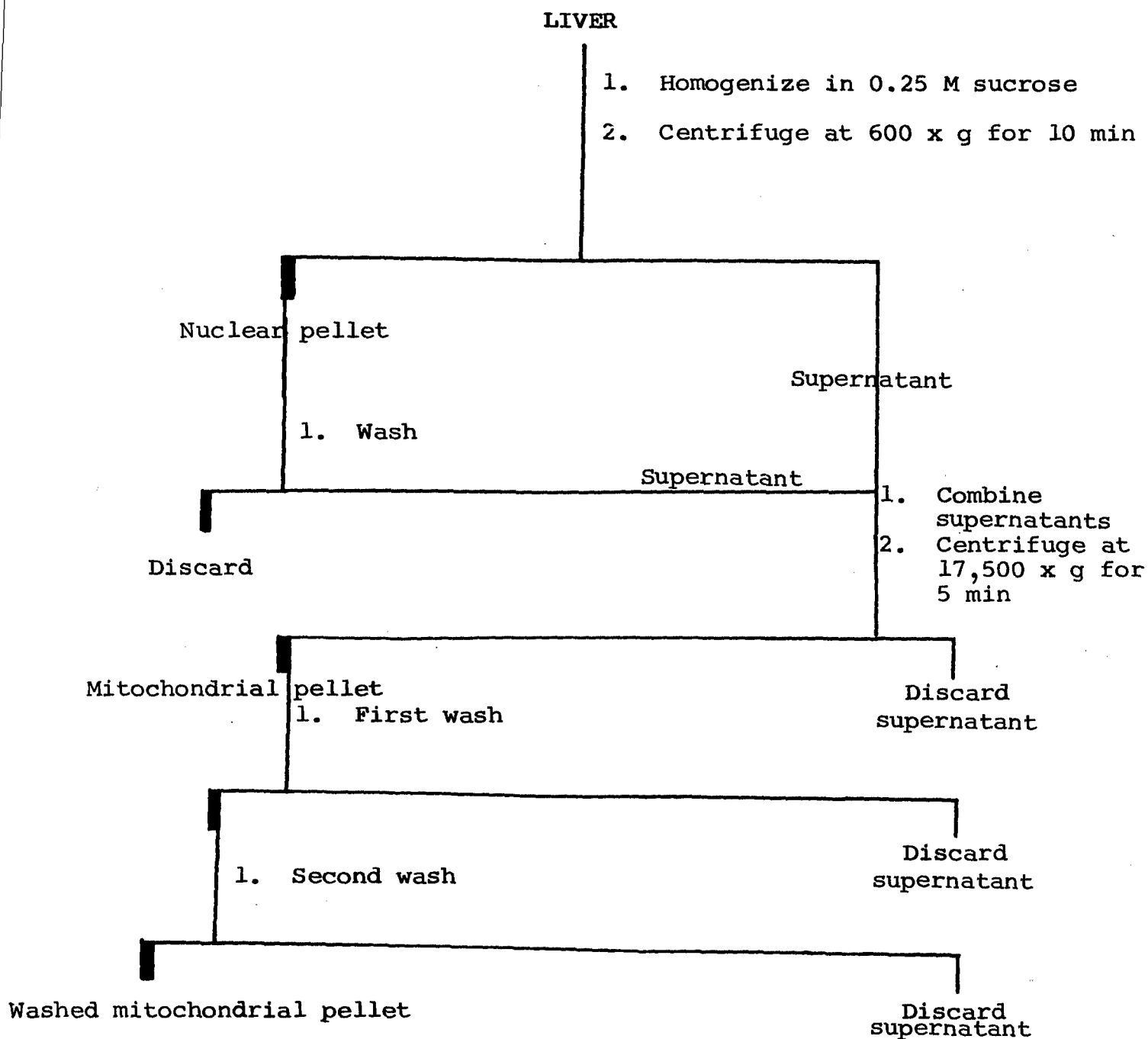
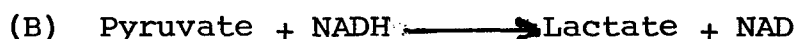
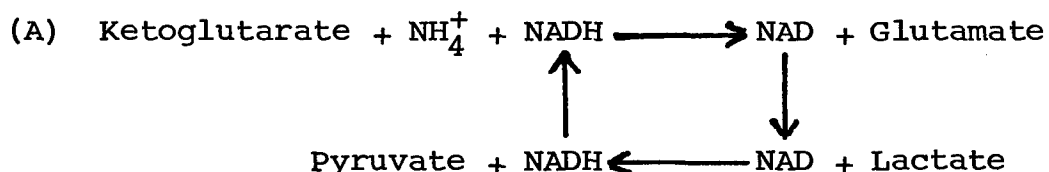


Figure 4. Flow diagram of procedure for liver mitochondrial fractionation.

## 2. NAD/NADH Ratio

Since conversion of oxidized NAD to its reduced form occurs rapidly within cells subjected to anaerobic conditions, valid analysis of these nucleotides can only be made when tissue cells in the intact animal are fixed to prevent increases of NADH levels due to ischemia. To accomplish this, the in situ freezing technique of Birch and Dippe (ref. 8) was carried out in lightly anaesthetized animals. NAD and NADH were extracted from the appropriate tissues in acid and basic media, respectively, and aliquots from each were analyzed separately by the enzyme cycling method of Lowry (ref. 9). This method provided the most sensitive means of quantitating NAD or NADH, since initial concentrations of either nucleotide were amplified 2500-fold during this procedure. The following scheme illustrates the overall reaction process:



In this scheme, (A) represents the enzymatic cycling method. After cycling for 0.5 hr at 30°C, pyruvate was formed 2500-fold for each original NAD or NADH. Lactic acid dehydrogenase is limited during cycling to prevent the formation of a large level of NADH, which might alter the kinetics to cause a back reaction to pyruvate. (B) represents the enzymatic assay of the pyruvate formed. Pyruvate is converted to lactate by the addition of a large excess of NADH and LDH. Decreases in the NADH level are measured spectrophotometrically at 340 mμ. The level of pyruvate formed during the cycling process was then related to standard preparations of nucleotide analyzed in a similar manner.

### 3. Arterial Blood Lactate/Pyruvate Ratio

The levels of lactate and pyruvate in arterial blood are quite sensitive and, like the cellular nucleotides just described, are subject to rapid changes relative to the state of aerobiosis. Blood was withdrawn rapidly from the abdominal aorta and treated immediately with acid to destroy the LDH activity in blood cells and the plasma. The levels of each metabolite were analyzed enzymatically with LDH and spectrophotometric readout by the method of Krasnow et al. (ref. 10). Calibration curves of standard lactate and pyruvate solutions were made to estimate all unknown sample preparations.

### 4. Total Mitochondrial LDH

The activity of LDH associated with liver and lung mitochondria, used in the respiration experiments, was determined by means of the Sigma method (ref. 11). This enzymatic assay is related to Wroblewski's spectrophotometric method but is carried out colorimetrically after the depletion of pyruvate substrate. The Sigma method provided a carefully standardized and convenient means for assaying multiple mitochondrial samples for simultaneous comparison.

### 5. Mitochondrial LDH Isoenzymes

LDH isoenzyme distributions were carried out electrophoretically in an agar-gel medium and the patterns developed with an incubating mixture containing lactate as substrate, NAD as a hydrogen acceptor, and nitro-blue tetrazolium dye as an electron acceptor. The reduced tetrazolium dye is deposited at the site of each isoenzyme migrant. The bulk of liver mitochondrial LDH is anaerobic and corresponds to band 5, which has the greatest cathodic migration.



### SECTION III

#### RESULTS

##### A. Normal Biochemical Values for Rats Breathing Air (Run N-1)

The objective of this experiment was to find the values for each of the biochemical analyses (Table V) for rats in a normal environment. The variances for each analysis between rats and experimental replicates were sought. Operation comparisons were not carried out since biological material was often limited and since a high degree of proficiency was required for each analysis.

To accomplish this objective, the following set of analyses was performed on rats exposed to ambient air (approximately 740 mm Hg) outside the test chamber.

In general, different rats were used for each analysis. Furthermore, each individual analysis was performed in duplicate. All liver and lung mitochondrial analyses were performed on rats sacrificed by decapitation. Lactate/pyruvate analyses were made on arterial blood obtained from separate rats. These rats were lightly anaesthetized with ether and blood withdrawn from the abdominal aorta. NAD/NADH values were obtained from separate rats by freezing the tissue before removal.

A total of up to 29 pairs of duplicate values was accumulated for the various biochemical analyses.

The literature values for rat liver mitochondrial oxygen consumption ( $Q(O/N)$ ) using  $\alpha$ -ketoglutarate as substrate range from approximately 30 to 50 (ref. 12 and 7). Our values ranged between 44 and 58, in agreement with those recently obtained by Lardy.

The values for P/O ratio found by Lardy are between 2.6 and 3.4 with an average of 3.2. Our average value was determined to be 2.89.

Rat arterial blood lactate was reported to be between 0.55 to 2.3 micromoles/ml by Newman (ref. 13), while work by Baker (ref. 14) indicates a level between 1.41 and 1.79 micromoles/ml. Our values range between 0.48 and 1.43 micromoles/ml. Pyruvate levels of 0.65 to 2.79 micromoles/ml have been reported by Everson (ref. 15), while our levels were 0.17 to 0.42 micromoles/ml.

Table V. Summary of normal biochemical values for rats breathing air

<u>Biochemical Analysis</u>	<u>Number Of Animals</u>	<u>Range</u>	<u>Mean (<math>\bar{x}</math>)</u>	<u>Standard Deviation</u>
Liver mitochondria				
Q (O/N)*	4	44.1-57.6	48.5	6.5
P/O ratio	13	2.60-3.19	2.89	0.16
LDH, B.B. units/mg N	4	349-1093	649	276
Lung mitochondria				
Q (O/N)**	2	81.6-111.2	96.4	20.9
LDH B.B. units/mg N	4	1929-2992	2655	333
Liver				
NAD, micromols/kg	28	330-1830	608	300
NADH, micromols/kg	29	29.7-239	77.8	40
NAD/NADH ratio	28	2.07-18.50	8.34	3.17
Lung				
NAD, micromols/kg	19	21-216	68	52
Arterial blood				
Lactate, micromols/ml	27	0.480-1.425	1.018	0.26
Pyruvate, micromols/ml	27	0.170-0.415	0.289	0.095
Lactate/pyruvate ratio	27	2.33-5.61	3.51	0.87

\* Microliters oxygen/10 min  
mg nitrogen

\*\* Microliters oxygen/60 min  
mg nitrogen

Values for mitochondrial LDH in both liver and lung were not found in the literature, thus no comparison can be made.

The NAD levels reported by Burch and Von Dippe (ref. 8) for the enzymatic cycling method averaged 339 micromoles/kg of liver and approximately 50 micromoles of NADH per kg. Our normal values averaged 608 micromoles of NAD and 78 micromoles of NADH per kg of liver. The average NAD/NADH ratio reported by these authors is between 10 and 20, while our range was determined to be between 3.1 and 18.5 with an average of 8.3.

Values for the lung nucleotides were not found in the literature.

#### B. Animals Exposed to Oxygen at One Atmosphere

The objective of the experimental exposures of animals to 1 atm of pure oxygen was to determine whether acute changes in cellular respiration precede ultimate mortality and to determine the phasing of such changes with morphological changes demonstrated in the earlier studies (ref. 3).

##### 1. Rats in the Felig-Lee Chamber and the Thomas Dome for Periods up to 96 hr

The experimental runs included exposure of rats to pure oxygen at approximately 1 atm pressure for periods up to 96 hr in the Felig-Lee chamber (runs 1, 2, 5, 6, and 14) and in the Thomas dome (runs 3 and 4). Exposures at 1 atm involving specialized techniques with regard to tissue removal were also carried out (run 15).

The Felig-Lee chamber and the Thomas dome differ with regard to airflow and air-lock operation. Periods of exposure with rats were chosen to correspond to morphological changes previously shown; similar changes in monkeys are delayed.

Runs 1 through 6 consisted of exposure of rats to pure oxygen in two different environmental chambers. In runs 1 and 2, they were exposed in the Felig-Lee chamber to oxygen for 6, 24, 48, and 72 hr. Only one set of animals breathing air outside the chamber was used for control, and they were analyzed in the interval between runs 1 and 2. In run 1, animals were removed from the chamber after 72 hr for recovery in air for a period of 10 days; in run 2, a similar recovery for a period of 3 days was made.

Runs 3 and 4 consisted of exposure of rats to pure oxygen in the Thomas dome for periods of 24, 48, 72, and 96 hr; control rats breathing air were used for analysis at the same time periods as the experimental animals.

Runs 5 and 6 were similar to runs 3 and 4 except that the Felig-Lee chamber was used.

All the biochemical analyses carried out required three groups of animals, designated U, V, and W (Table IV). In the experimental subgroups (for each period of exposure), 3 to 4 animals were used. The control subgroups consisted of 2 animals each.

#### a. Liver Mitochondrial Q(O/N) and P/O Ratio

An analysis of variance of the liver mitochondrial Q(O/N) values showed no significance with respect to the effect of oxygen.

The results of analyses for P/O ratio on liver mitochondria from runs 1 through 6 are shown in Table VI. Duplicate analyses were obtained for each rat. The ranges shown span all the values for each group.

In general, the data show significant reduction in P/O ratio with exposure to oxygen in the chamber. In runs 1 and 2, some of the animals that had been exposed to oxygen in the Felig-Lee chamber for 72 hr were removed from the chamber and exposed to air. Nearly complete return of P/O ratios to normal values was observed in 3 days. Additional experiments are required to confirm this observation. The reduction in P/O ratio due to oxygen exposure in the Thomas dome (runs 3 and 4) appears to be less than that in the Felig-Lee chamber (runs 1, 2, 5, and 6).

The mean differences between the experimental P/O ratios and the air control ratios are plotted in Figure 5. The control pertinent to each run, namely, the joint control for runs 1 and 2, and the separate control for each exposure period in runs 3 through 6 is used as the zero reference point. In runs 1, 2, 5, and 6 all differential values are negative, showing a reduction in P/O ratio by oxygen exposure. Reduction as a function of time is suggested but not uniform in these runs. Good recovery in either 3 or 10 days upon reexposure to air is shown in runs 1 and 2. In runs 3 and 4 the differential plots show trends that tend to parallel, but they may not be significantly different from the controls.

Table VI. Comparison of liver mitochondrial P/O ratios of rats exposed to air, oxygen, and air after oxygen at approximately one atmosphere pressure in two environmental chambers

Run	Chamber	Atmosphere	6 Hr		24 Hr		48 Hr		72 Hr		96 Hr		3 Days		10 Days	
			Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range
1	None Felig-Lee	Air <sup>b</sup> O <sub>2</sub>	3.12 <sup>b</sup> 2.74 -0.38	2.86-3.28 2.45-3.14	3.12 <sup>b</sup> 2.72 -0.40	2.02-3.06	3.12 <sup>b</sup> 2.60 -0.52	1.41-2.76	3.12 <sup>b</sup> 2.62 -0.50	2.43-2.76					3.12 <sup>b</sup>	
		Air after O <sub>2</sub> for 72 hr														2.84 -0.28
2	None Felig-Lee	Air <sup>b</sup> O <sub>2</sub>	3.12 <sup>b</sup> 2.83 -0.29	2.69-2.89	3.12 <sup>b</sup> 2.84 -0.28	2.16-3.21	3.12 <sup>b</sup> 2.82 -0.30	2.45-3.17	3.12 <sup>b</sup> 2.17 -0.95	1.99-2.44			3.12 <sup>b</sup>		2.86 -0.26	2.61-3.16
		Air after O <sub>2</sub> for 72 hr														
3	None Thomas dome	Air O <sub>2</sub>	2.94 3.01 +0.07	2.87-3.04 2.67-3.35	3.01 3.42 +0.41	2.67-3.35 3.30-3.50	2.62 3.40 +0.78	2.27-2.88 3.09-3.59	2.79 3.74 -0.31	2.56-2.99 3.64-3.85	2.73 3.53 -0.18	2.65-2.86 3.27-3.58				
4	None Thomas dome	Air O <sub>2</sub>	3.35 3.27 -0.08	3.22-3.58 3.21-3.33	3.35 3.27 -0.08	3.22-3.58 3.21-3.33	2.86 2.79 -0.07	2.78-2.97 2.48-2.90	2.68 2.02 -0.66	2.49-2.91 1.67-2.32	2.84 2.32 -0.52	2.74-2.94 2.12-2.56				
5	None Felig-Lee	Air O <sub>2</sub>	3.38 2.86 -0.52	2.94-3.67 2.75-2.97	3.38 2.86 -0.52	2.94-3.67 2.75-2.97	3.16 2.84 -0.32	3.00-3.23 2.70-3.02	3.11 2.82 -0.29	2.98-3.24 2.52-3.16	3.10 2.50 -0.60	2.99-3.23 2.40-2.60				
6	None Felig-Lee	Air O <sub>2</sub>	3.35 3.27 -0.08	3.22-3.58 3.21-3.33	3.35 3.27 -0.08	3.22-3.58 3.21-3.33	2.86 2.79 -0.07	2.78-2.97 2.48-2.90	2.68 2.02 -0.66	2.49-2.91 1.67-2.32	2.84 2.32 -0.52	2.74-2.94 2.12-2.56				

<sup>a</sup>The pressures were 760 mm Hg for oxygen in the Felig-Lee chamber, 750 mm Hg for oxygen in the Thomas dome, and ground level pressure for air.

<sup>b</sup>A common set of air control animals was used for each experimental exposure in runs 1 and 2.

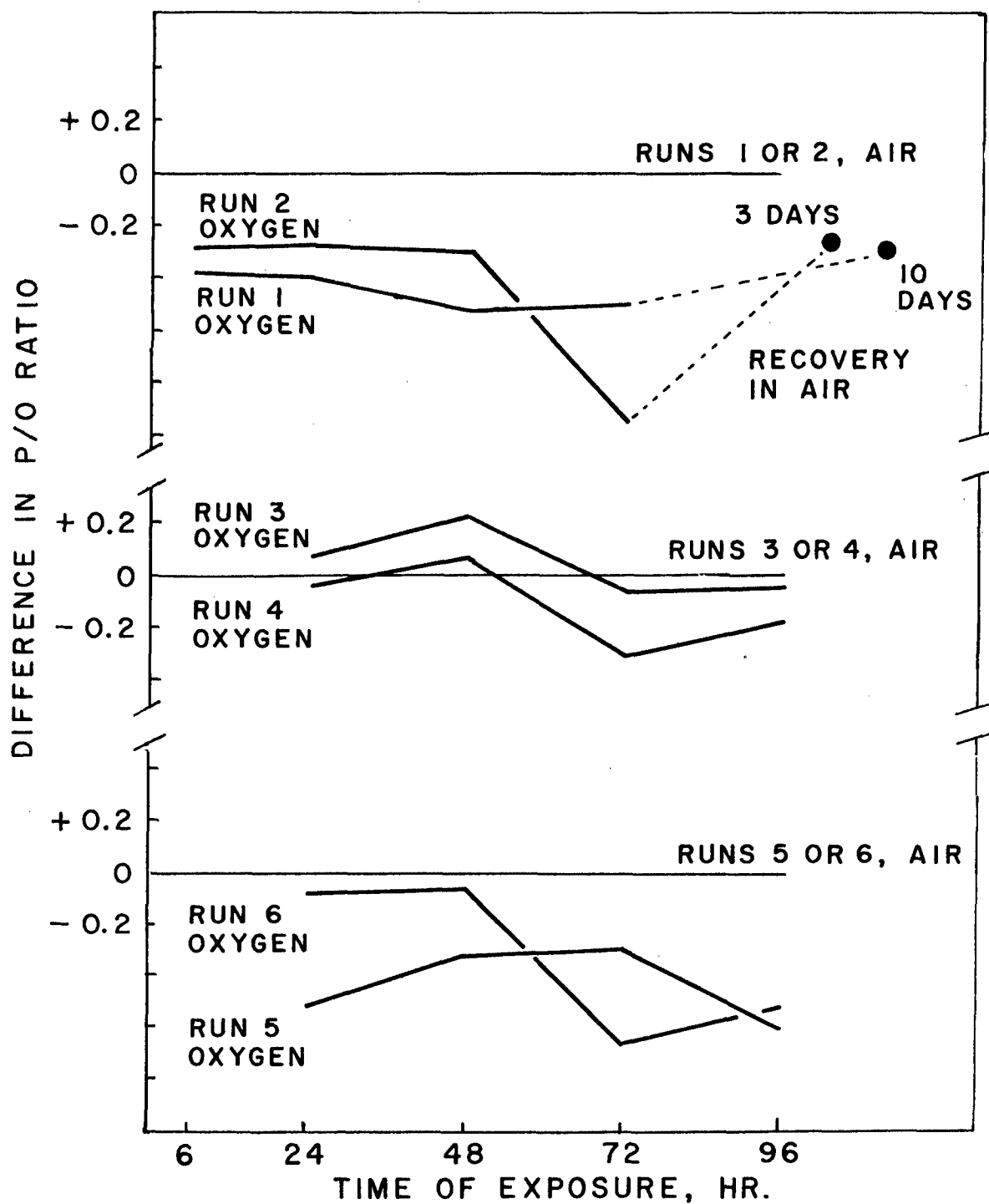


FIG. 5 COMPARISON BY INDIVIDUAL RUN OF CHANGE IN LIVER MITOCHONDRIAL P/O RATIOS FOR RATS EXPOSED TO 1 ATM OF OXYGEN WITH THAT FOR RATS EXPOSED TO AMBIENT AIR.

A summary of an analysis of variance for liver mitochondrial P/O ratios for runs 5 and 6 is shown in Table VII. The analysis is done on the basis of log P/O ratio. The effect O is the overall effect of oxygen treatment, and T x O interaction measures the change of the oxygen exposure effect with time. R x O and R x T x O indicate the variation of the above effects from run to run. Since neither of these interactions is significant, the test with respect to residual error is used. The results show a significant control versus treatment effect. The importance of the simultaneous controls is demonstrated by size of the R, T, and R x T effects. These are design variables that are necessary for controlling irrelevant variation.

#### b. Lung Mitochondrial Q(O/N)

Lung mitochondrial respiration studies were carried out on all animals employed in the oxygen exposure experiments at 1 atm. The succinate Q(O/N) was determined in each of the control and experimental animals. The values did not show any significant variance with respect to oxygen treatment. It should be pointed out, however, that succinate may not be suitable for the most rigorous evaluation of lung mitochondrial respiration.

#### c. Liver Mitochondrial LDH

The results of analyses for LDH activities on liver mitochondria from runs 1 through 6 are shown in Table VIII. The data are from the same animals used for the P/O studies (Table VI).

Considerable elevation of LDH values corresponding to the oxygen-exposed animals was found in runs 1 and 2 (Figure 6). Air control animals were common to these two runs and not analyzed simultaneously with each time of oxygen exposure. Hence the extent of an oxygen effect is unknown.

In runs 3 and 4 little if any significance can be attached to either the oxygen effect or the time of exposure.

The air control values paralleled the values of the oxygen-exposed animals in both run 5 and run 6. However, the response patterns of the two runs differed with respect to time. These trends are shown in Table VIII.

Table VII. Analysis of variance of liver mitochondrial  
P/O ratios from runs 5 and 6

<u>Effect</u>	<u>d.f.</u>	<u>Sum of Squares</u>	<u>Mean Square</u>	<u>F</u>	<u>P</u>
Run (R)	1	0.00673	0.00673	2.246	n.s.
Time (T)	3	0.04627	0.01542	5.146	0.99
Oxygen (O)	1	0.02448	0.02448	8.170	0.99
R x T	3	0.02566	0.00855	2.853	0.93
R x O	1	0.00063	0.00063	0.210	n.s.
T x O	3	0.01094	0.00365	1.218	n.s.
R x T x O	3	0.00822	0.00274	0.914	n.s.
Error	<u>20</u>	<u>0.05993</u>	0.00300		
Total	35	0.18286			



Table VIII. Comparison of liver and lung mitochondrial LDH values of rats exposed to air, oxygen and air after oxygen at approximately one atm in two environmental chambers

Run	Chamber	Atmosphere	6 Hr	24 Hr	48 Hr	72 Hr	96 Hr	3 Days	10 Days
			Avg.	Range	Avg.	Range	Avg.	Range	Avg.
1	None Felig-Lee	Air O <sub>2</sub>	553 1291 +748	186-820 992-1722 +666	553 2247 +1694	186-820 1862-2635 +3614	553 4167 +3614	186-820 3857-4455	553 918 +365
		Air after O <sub>2</sub> for 72 hr							
2	None Felig-Lee	Air O <sub>2</sub>	553 351 -202	186-820 267-1129 -10	553 953 +400	186-820 672-1249 +2027	553 2580 +2027	186-820	
		Air after O <sub>2</sub> for 72 hr							
3	None Thomas dome	Air O <sub>2</sub>	1352 1271 -81	940-1745 1102-1432 -81	1128 1572 +444	963-1379 1182-1887 +321	1477 1798 +321	1346-1598 1500-2002	1200 1446 +246
		Air O <sub>2</sub>	1463 2001 +538	1396-1558 1393-2811 +258	1172 1430 +258	847-1495 1171-1758 +484	1117 1601 +484	985-1272 1305-1981	1462 1599 +137
4	None Thomas dome	Air O <sub>2</sub>							
5	None Felig-Lee	Air O <sub>2</sub>	1330 1108 -222	1025-1581 848-1421 -222	350 751 +401	175-430 690-954 +674	641 1315 +674	368-862 1225-1459	----- ----- -----
6	None Felig-Lee	Air O <sub>2</sub>	1212 682 -530	1044-1380 533-1011 -530	1471 1514 +43	891-2032 1407-1679 +510	2145 2655 +510	2105-2199 437-720 868-1913	606 1339 +733

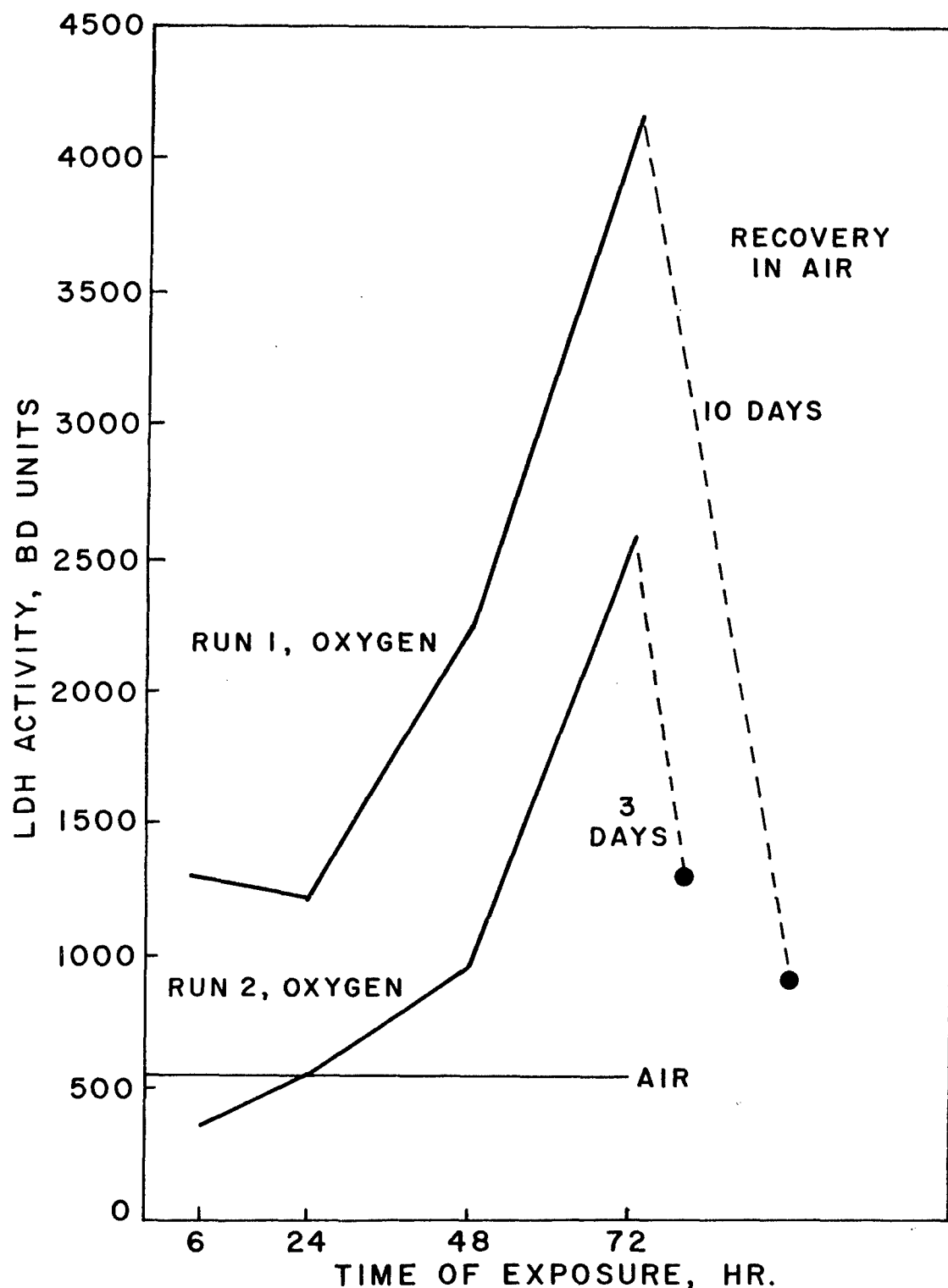


FIG. 6 COMPARISON IN RUNS 1 AND 2 OF CHANGE IN LIVER MITOCHONDRIAL LDH ACTIVITY FOR RATS EXPOSED TO 1 ATM OF OXYGEN WITH THAT FOR RATS EXPOSED TO AMBIENT AIR.

The differential plots between air controls and oxygen-exposed animals for runs 3 through 6 are shown in Figure 7. As has been indicated, the rise in LDH values with time of exposure in runs 1 and 2 may not be solely an oxygen effect. No significant oxygen effect was noted in runs 3 and 4. In runs 5 and 6, a 24-hr depression in response, followed by a gradual rise due to oxygen exposure, was noted at subsequent time intervals.

#### d. Liver Mitochondrial LDH Isoenzyme Distributions

Liver mitochondrial LDH isoenzyme distributions were determined in several rats that demonstrated acute increases in total mitochondrial LDH activity after exposure to 1 atm of pure oxygen for periods of 6 and 72 hr in the Felig-Lee chamber during run 1 (Figure 6). The patterns suggested a decrease in the small amount of aerobic isoenzyme (band 1 and 2) after oxygen exposure. Even the trace of intermediate isoenzyme (neither aerobic nor anaerobic, band 3) is substantially reduced, particularly in the 6-hr exposed rats. The alterations observed in these patterns could reflect changes that were caused by the manipulation of mitochondria during isolation procedures, i.e. elution of isoenzymes during washing of mitochondria. However, the mitochondrial isolation procedure was carried out uniformly and precisely for each rat liver preparation. If only the effect of oxygen exposure is considered, the alteration would indicate that LDH produced consists almost entirely of the anaerobic type of subunit (band 5).

It is of interest that rat liver homogenate has larger quantities of the aerobic bands (1 and 2) than the corresponding mitochondria. The relation of homogenate to mitochondrial LDH will be further investigated.

Previous experience with measurement of serum and tissue LDH activities in gamma-radiation animals at IIT Research Institute (ref. 16) has shown that a doubling or tripling of value is generally required for apparent significance for individual animals. Hence, we tentatively conclude that no significant effect of oxygen was found with respect to distribution of isoenzymes in liver mitochondrial LDH.

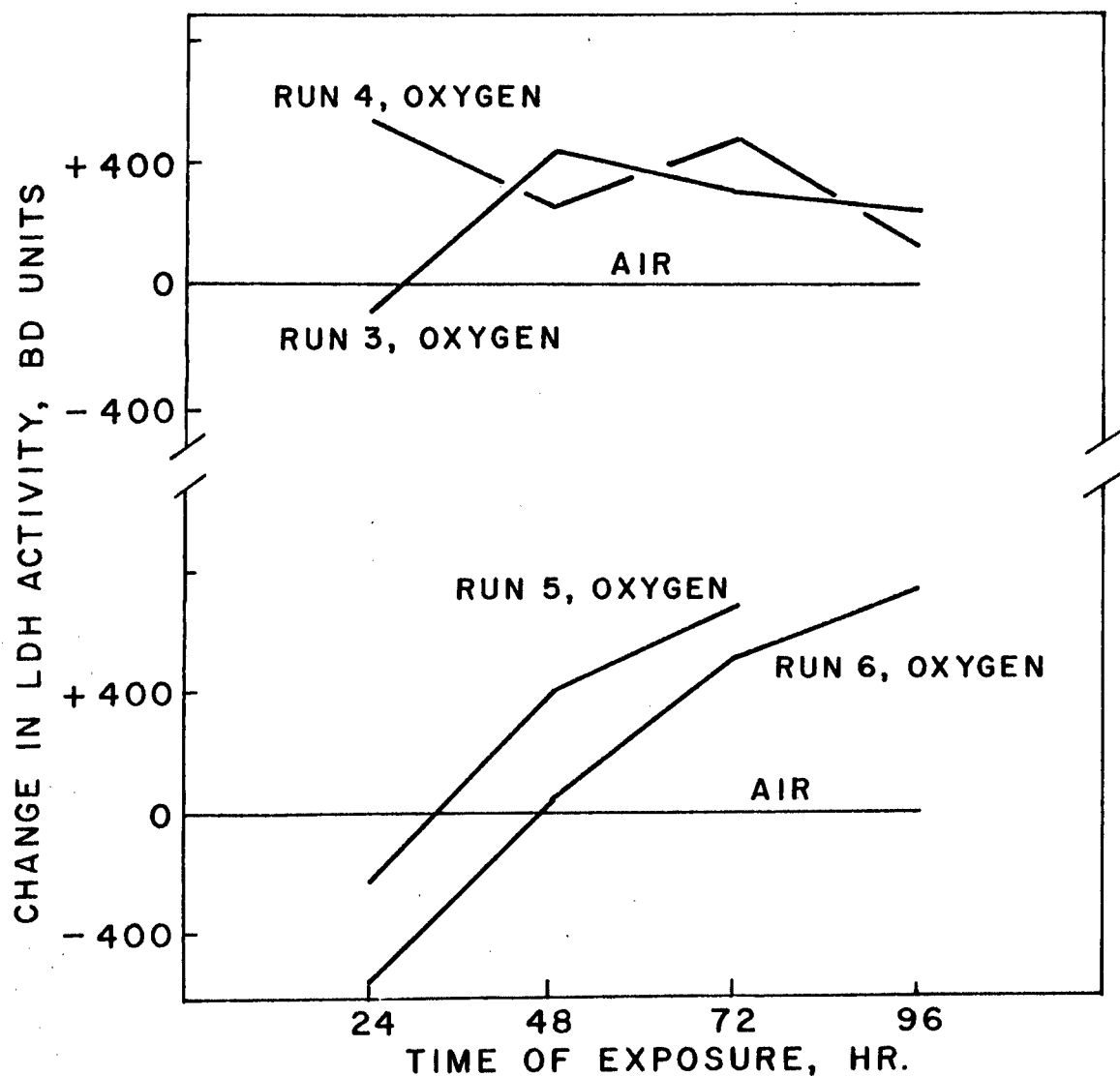


FIG. 7 COMPARISON IN RUNS 3,4,5 AND 6 OF CHANGE IN LIVER MITOCHONDRIAL LDH ACTIVITY FOR RATS EXPOSED TO 1 ATM OF OXYGEN WITH THAT FOR RATS EXPOSED TO AMBIENT AIR.

#### e. Lung Mitochondrial LDH

The lung mitochondrial LDH values showed considerably more scatter than those for liver mitochondria. Hence a presentation of this data is not considered fruitful.

#### f. Weight Change

In addition to the biochemical indexes, change in animal weight was observed initially during exposure to oxygen. Since weight data had been accumulated for all animals thus exposed, addition of this criterion as an index of oxygen toxicity was possible. Change in weight reflects the overall anabolic or catabolic state of the animal and hence is a general physiological index.

Weight changes in runs 1 and 2 are not presented since control animals were not used simultaneously with the exposed animals.

The percent weight changes of rats during exposure to air or oxygen in runs 3, 4, 5, and 6 are shown in Table IX. In general, the results show a reduction in rate of weight gain (including weight loss) during oxygen exposure in comparison with the gain of control animals exposed to air. The mean difference of all runs increased from -2.0% at 24 hr to -25.4% at 96 hr. Hence the change in rate of weight gain is a useful gross physiological indication of the effects of exposure to oxygen at 760 mm Hg. No significant difference was found in the mean differences of runs 3 and 4 in comparison with those of runs 5 and 6. It is evident that the weight change index showed similar physiological impairment as a result of oxygen exposure in either the Thomas dome or the Felig-Lee chamber.

#### g. Analyses of Variance

Analyses of variance were performed on each of the following variables from runs 3 through 6 (Table X):

- (1) Q(O/N) of liver mitochondria
- (2) P/O ratio of liver mitochondria
- (3) LDH of liver mitochondria
- (4) LDH of lung mitochondria

Table IX. Percent weight change of rats during exposure to air or oxygen in runs 3 through 6

Run No.	Atmosphere	% Change in Rat Weight <sup>a</sup>				
		Time, hr				
		24	48	72	96	Mean
3	Air	+0.7	+ 9.5	+12.9	+16.4	+ 9.80
	Oxygen	+1.8	+ 6.1	+ 1.6	- 3.8	+ 1.40
4	Air	+3.8	+ 9.6	+16.7	+20.6	+12.70
	Oxygen	-4.1	+10.6	- 3.7	- 5.4	- 0.70
3	Air to oxygen change	+1.1	- 3.0	-11.3	-20.6	- 8.45
4	Air to oxygen change	-7.9	+ 1.0	-20.4	-26.0	-13.32
5	Air	+9.2	+12.6	+12.1	+17.3	+12.80
	Oxygen	+2.9	+ 5.2	+ 1.9	- 3.6	+ 1.60
6	Air	+3.1	+ 7.4	+21.8	+24.0	+14.08
	Oxygen	+8.6	+ 3.0	- 4.5	- 9.9	- 0.70
5	Air to oxygen change	-6.3	- 7.4	-10.2	-20.9	-11.20
6	Air to oxygen change	+5.5	- 4.4	-26.3	-33.9	-14.78
Mean Difference		-2.0	- 3.5	-17.1	-25.4	-12.00

<sup>a</sup>Plus sign designates increase in weight due to oxygen exposure; minus sign designates decrease in weight due to oxygen exposure.

Table X. Summary of analyses of variance for runs 3 through 6 in which rats were exposed to 1 atm of oxygen

No.	Variable Name	Level of Statistical Significance <sup>a</sup>		
		Oxygen Effects	Time x Oxygen	Run x Oxygen
1	Q(O/N) of liver mitochondria	n.s.	n.s.	n.s.
2	P/O ratio of liver mitochondria	0.01	0.05	0.05
3	LDH of liver mitochondria	0.001	0.01	n.s.
4	LDH of lung mitochondria	n.s.	0.10	0.01
5	NADH of liver	n.s.	n.s.	n.s.
6	NAD of liver	0.001	n.s.	0.001
7	NAD/NADH ratio for liver	n.s.	n.s.	n.s.
8	Lactate in blood	0.05	n.s.	0.20
9	Pyruvate in blood	0.05	n.s.	0.10
10	Lactate/pyruvate ratio for blood	n.s.	n.s.	n.s.
11	Weight loss	0.001	0.001	n.s.
				0.001

<sup>a</sup>n.s. indicates not significant. Significance levels exceeding 0.05 are not considered significant but are included to indicate trends.

- (5) NADH of liver
- (6) NAD of liver
- (7) NAD/NADH ratio for liver
- (8) lactate in blood
- (9) pyruvate in blood
- (10) lactate/pyruvate ratio for blood
- (11) weight loss.

Variables 2, 3, 6, 8, 9, and 11 show a difference in the oxygen effect for the animals exposed to 1 atm of oxygen compared with the room air controls; this was significant when compared with the random variation in the data. Variables 2, 3, and 11 showed significant variation of the oxygen effect with time. Run-to-run variation in the level of the oxygen toxicity effect leaves some doubt concerning the reproducibility of the oxygen effect for variables 2, 6, 8, and 9.

The trends of variables 2, 3, and 11 with respect to time are shown in Figures 8, 9, and 10. The 95% confidence interval for the P/O ratio of animals exposed to oxygen was completely outside the range for the animals exposed to air after 72 hr; it remained unchanged at 96 hr. Hence the change at this time period appears significant. The liver mitochondrial LDH values were significantly higher after 48 hr of exposure to oxygen. However, the elevation was not as great as that in runs 1 and 2. The effect on change in weight was greater than either of the other two indexes. It was significantly reduced at 72 hr of exposure and considerably lower at 96 hr.

The confidence limits for the control groups are generally larger than those for the experimental animals, since fewer control animals were used. The confidence limits were calculated on the basis of the number of samples used at each time period.

The establishment of the reproducibility of these results was not the primary objective of this experimental unit (runs 3, 4, 5, and 6). More important were the identification of trends for gaining insight into the mechanisms of oxygen toxicity and a broad screening of variables that could be useful



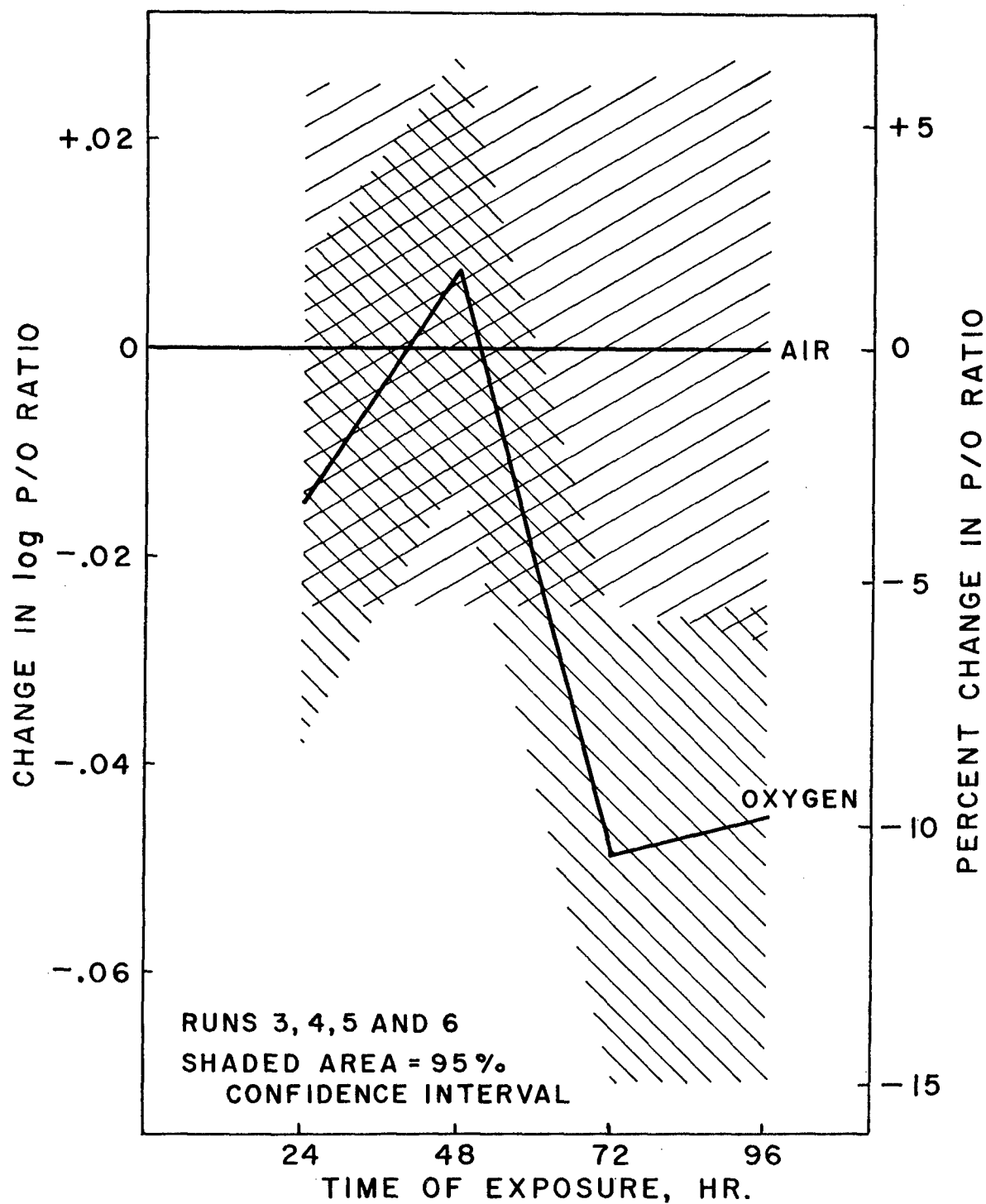


FIG. 8 STATISTICAL COMPARISON OF CHANGE IN LIVER MITOCHONDRIAL P/O RATIO FOR RATS EXPOSED TO 1 ATM OF OXYGEN WITH THAT FOR RATS EXPOSED TO AMBIENT AIR.

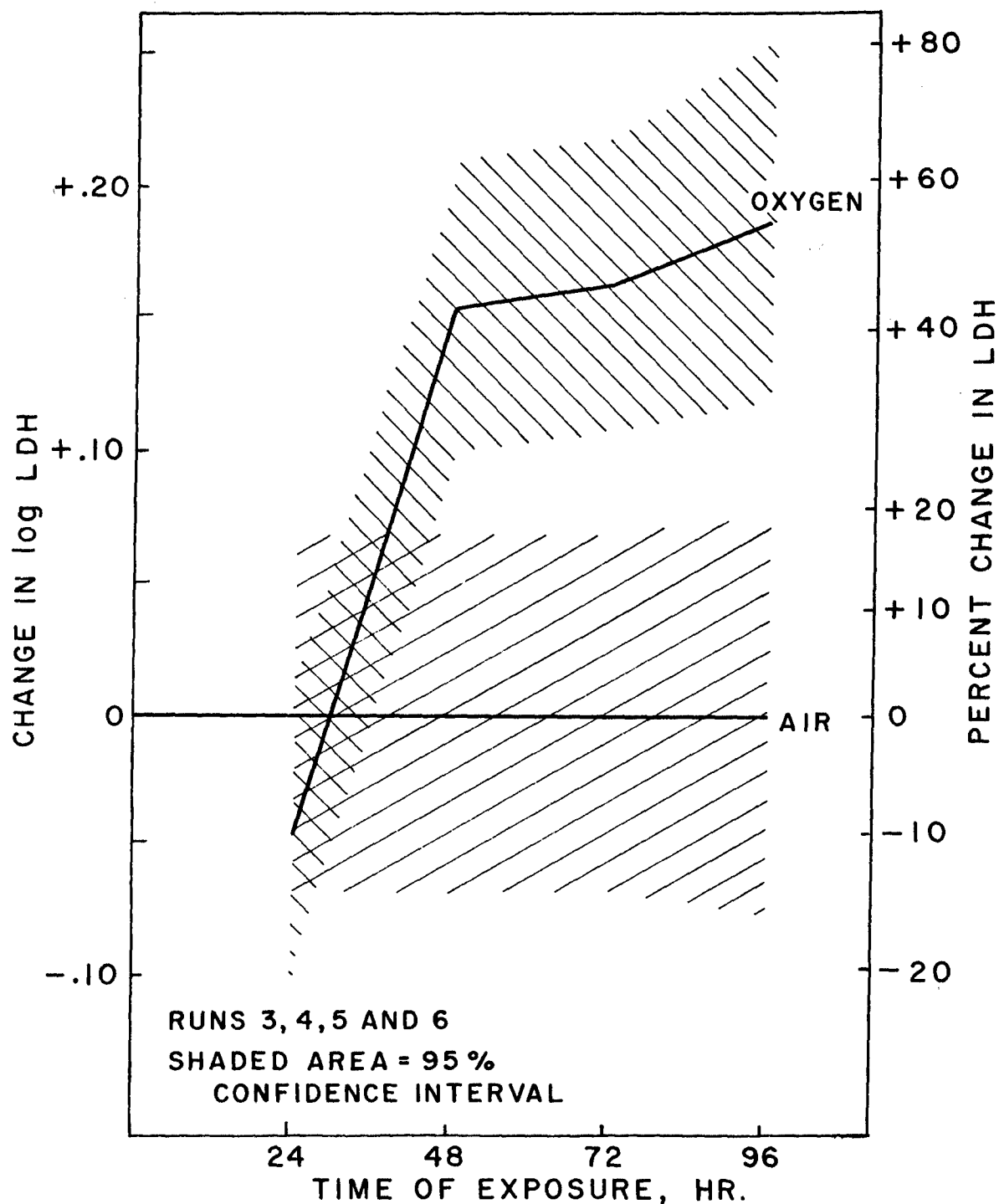


FIG. 9 STATISTICAL COMPARISON OF CHANGE IN LIVER MITOCHONDRIAL LDH FOR RATS EXPOSED TO 1 ATM OF OXYGEN WITH THAT OF RATS EXPOSED TO AMBIENT AIR.

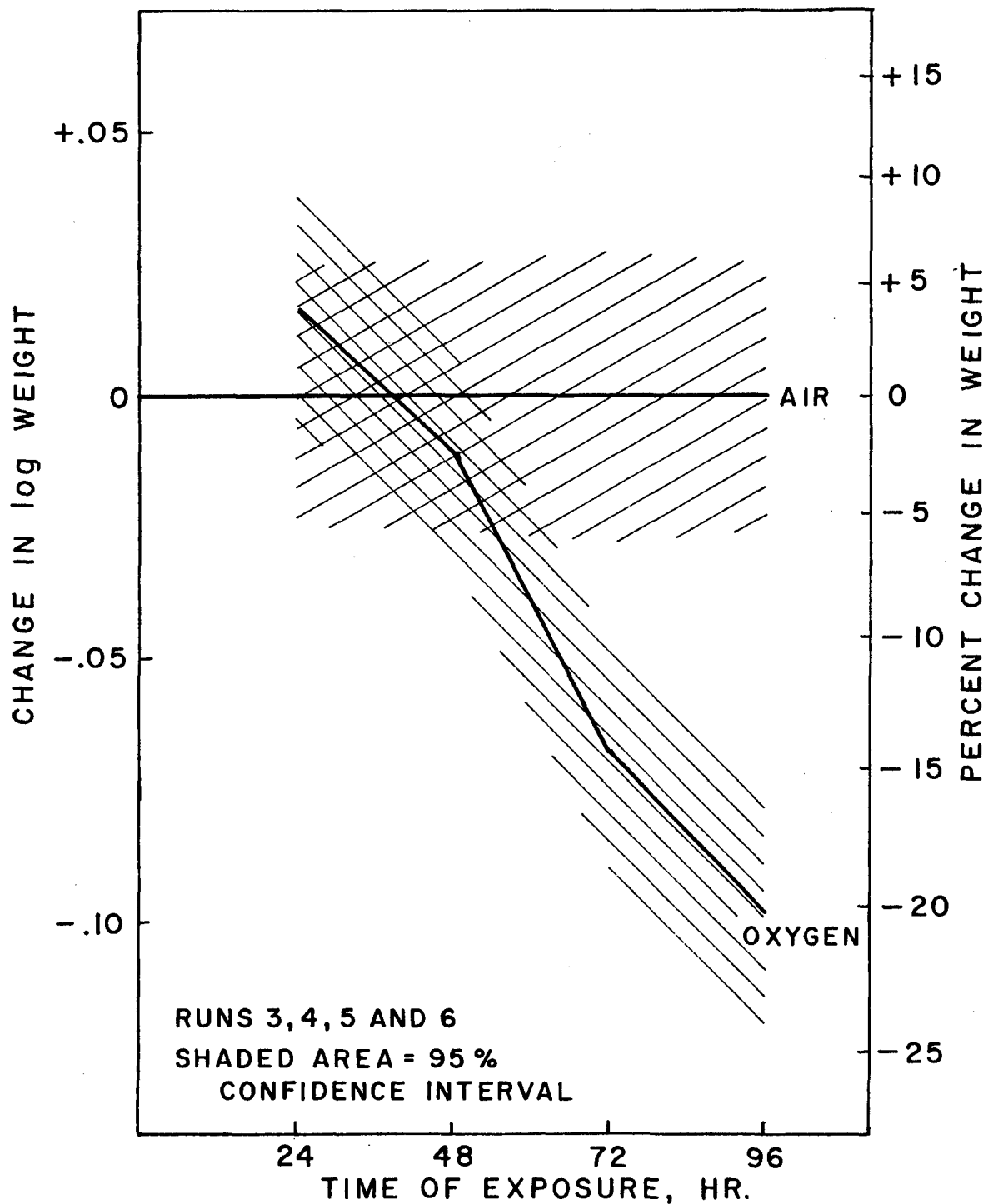


FIG. 10 STATISTICAL COMPARISON OF CHANGE IN WEIGHT FOR RATS EXPOSED TO 1 ATM OF OXYGEN WITH THAT FOR RATS EXPOSED TO AMBIENT AIR.

in the detection of effects for less severe, 1/3 atm oxygen exposure. To this end, we turned our attention to the derivation of an index that is most sensitive to typical oxygen toxicity effects.

This was accomplished by an analysis of covariance involving regression on an arbitrary index of oxygen exposure. The resulting regression coefficients give an index combining several variables observed on the same animals. Not all of the above variables were determined for each animal. Animals for which variables 1, 2, 3, 4, and 11 are observed in parallel (animal group U) were analyzed. Only P/O ratio and weight change contributed significantly to the regression. Variable 3, LDH of liver mitochondria, although showing a significant effect in univariant analysis, has an effect that is largely correlated with the P/O ratio or weight change variables. The relationship of the partition of the sums of squares resulting in this phenomena is indicated in Figure 11.

The resulting index of oxygen exposure can be simplified to the form:

$$\log W_2/W_1 + 1/5 \log P/O$$

where  $W_1$  and  $W_2$  are the initial and terminal weight, respectively.

## 2. Monkeys in the Thomas Dome for Periods up to 170 hr

Male and female Rhesus (Macacca mulata) monkeys were exposed to 1 atm of pure oxygen for periods of 24, 48, 72, 96, 120, 144 and 170 hr. One monkey was removed after each of the above time periods, and a small section of liver was removed surgically under Nembutal anaesthesia and immediately frozen in liquid Freon. The tissue was analyzed in the usual manner for both NAD and NADH. Analytical results for each of the time periods were not treated statistically since adequate control values were not analyzed concurrent with the experiment. The level of NAD ranged between 280 and 380 micromoles/kg of tissue as compared with an average value of 410 micromoles/kg in the case of the control monkey. On the other hand, the experimental values of NADH ranged between 37 to 67 micromoles/kg of frozen tissue compared with 73 micromoles/kg for the control. The scatter was considerably greater in regard to NADH, particularly at 48 hr. No significance is readily apparent since only two animals were used in each time period.

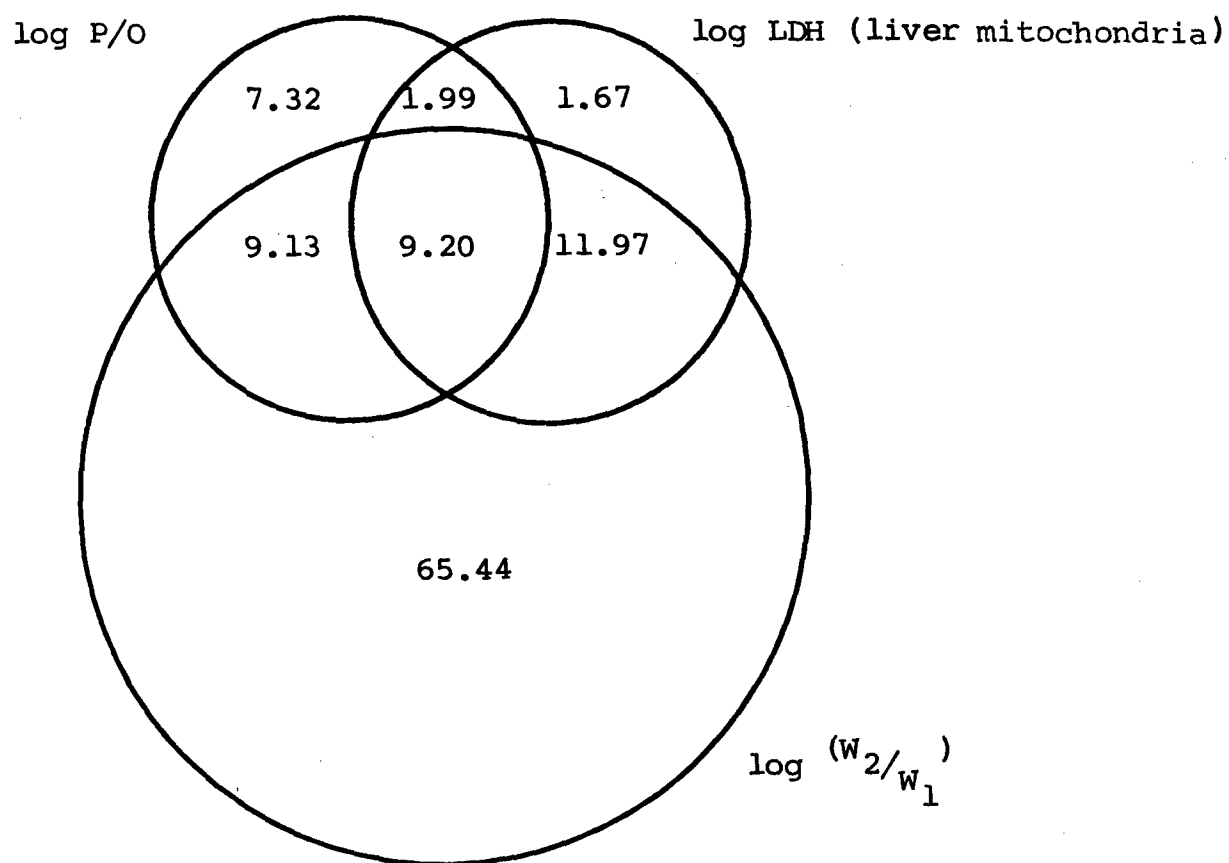


Figure 11. Partition of the sums of squares showing the common parts of variance related to an oxygen exposure index.

### C. Animals Exposed to Oxygen at One-Third Atmosphere

The objective of the experimental exposures of animals to 1/3 atm of pure oxygen was to determine whether biochemical changes could be detected in animals exposed to atmospheres simulating those of manned space flight. Several biochemical indices sensitive to the effects of excess oxygen were found in animals exposed to oxygen at 1 atm. If the mechanism of any more chronic oxygen impairment at 1/3 atm is similar to the acute changes at 1 atm, these same indices should be applicable at the reduced pressure. Morphological changes in liver and kidney cells have been observed in rats after 7 to 14 days exposure to 1/3 atm that were similar to those found after 1 to 3 days exposure to 1 atm. Hence any metabolic changes at the subcellular level should have particular significance.

All experiments with exposure to oxygen at 1/3 atm were carried out in the Thomas dome. The rat experiments consisted of 2 runs for periods up to 2 weeks (runs 12 and 13) and 1 run for approximately 8 months that included an air recovery of 38 days (run 10). For the short exposure periods (runs 12 and 13), rats were the Sprague Dawley strain from the same supplier (Harlan), and for the prolonged exposure (run 10), from another supplier (Carworth). The biochemical analyses were carried out in the usual manner. The P/O data and the weight data were subjected to an analysis of variance for comparison with similar analyses of data obtained at 1 atm of oxygen.

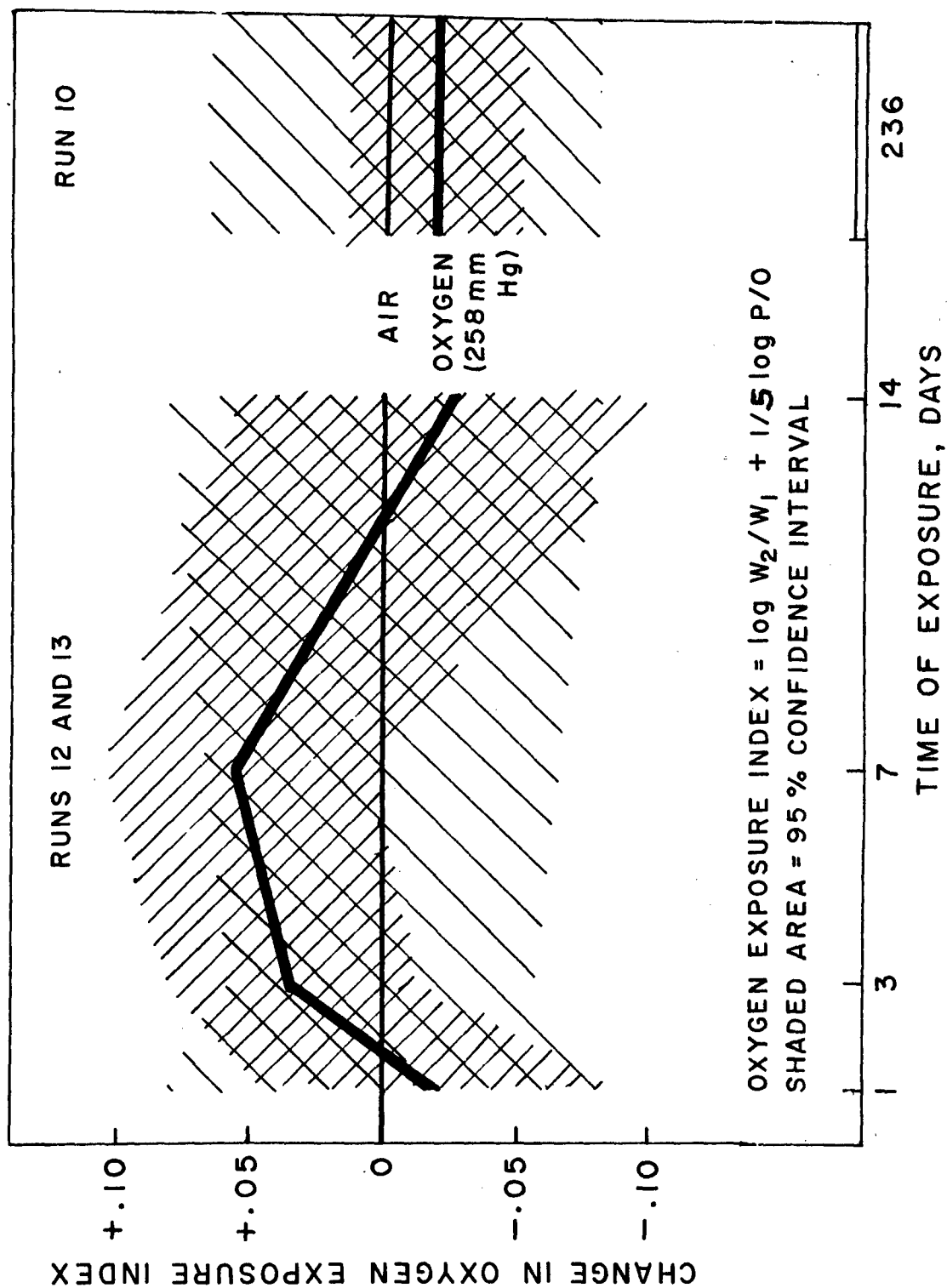
Large animal experiments included run 8 with dogs and runs 9 and 11 with monkeys.

#### 1. Rats in the Thomas Dome for Periods up to 236 Days (Runs 10, 12, and 13)

Rats were exposed to oxygen at 1/3 atm in three runs for 24, 72, 168, and 336 hr; 236 days, and a recovery period of 38 days in air. The index obtained for rats at 1 atm, namely,

$$\log W_2/W_1 + 1/5 \log P/O$$

was used to test for an oxygen effect at 1/3 atm. The results are shown in Figure 12. An oxygen toxicity effect cannot be established from this scanty data; however, an interesting trend is evidenced. The upward trend indicated in the graphs would suggest actually improved conditions for the animals. Later in



**FIG. 12** CHANGE IN OXYGEN EXPOSURE INDEX OF RATS BREATHING PURE OXYGEN AT 258 mm Hg PRESSURE IN COMPARISON TO RATS EXPOSED TO AMBIENT AIR.

the exposure it would appear that the animals level off very near the normal mean, suggesting actual adaptation. Further experimentation is required to establish this hypothesis. From these results, it would appear that the mechanism of oxygen effect for 1/3 atm of pure oxygen, if it exists, is fundamentally different from that shown in 1 atm of oxygen. Hence, more broad screening of variables may be required, in addition to larger experiments.

It is of interest that the index log P/O plotted in the same manner shows an almost identical pattern. Hence weight change made no significant contribution to this response. In this respect the response at 1/3 atm with respect to these two indexes, although not as extensive, had an entirely different pattern qualitatively than that obtained at 1 atm of pure oxygen. The use of a different supplier of rats enters into the calculation only at the terminal value of 236 days.

Multivariant analysis by utilizing repeated determinations of several variables, which have been made on larger animals, may lead to more sensitive indicators of oxygen toxicity.

The P/O ratio did not significantly change upon exposure to animals to air for 38 days after oxygen exposure. No significant changes in other indexes were found (blood lactate and pyruvate were not analyzed).

In view of the unusual response observed in P/O ratio and the importance of this simulation of the manned space cabin, additional experiments are planned to search for additional biochemical indexes.

## 2. Dogs in the Thomas Dome for 236 Days

An inbred strain of beagle was exposed to pure oxygen at 258 mm Hg in the Thomas dome for 236 days. Two dogs were exposed to oxygen in the dome while two dogs breathed air outside the dome for use as simultaneous controls. The biochemical analyses consisted of liver mitochondrial Q(O/N), P/O, and liver NAD/NADH. Samples were fractionated immediately for mitochondria; bits were also frozen in liquid Freon for nucleotide analysis in the usual manner. The results of these analyses are summarized in Table XI.



The average control and experimental P/O ratios were 2.47 and 2.26, respectively. These values are not significantly different, and both are lower than the normal values for rat liver (approximately 3). All NAD/NADH values ranged from 5.5 to 15.3, with no apparent difference due to oxygen exposure.

Table XI. Liver mitochondrial P/O ratios and liver NAD/NADH ratios for dogs exposed in the Thomas dome to oxygen at 258 mm Hg (run 8)

<u>Condition</u>	<u>P/O</u>	<u>NAD/NADH</u>
Air control	2.69	15.3
	2.09, 2.41	7.8
Avg.	2.47	11.55
Oxygen, 236 days	2.16, 2.25	5.5
	2.16, 2.47	10.6
Avg.	2.26	8.05

### 3. Monkeys in the Thomas Dome for Periods up to 236 Days with 40 Day Air Recovery (runs 9 and 11)

Monkeys were exposed to pure oxygen at 258 mm Hg pressure in the Thomas dome for 236 days in run 9. Two monkeys were exposed to oxygen in the dome while two were used in air outside the dome for controls. Liver tissue excision for P/O ratio and nucleotide analyses were the same as described for dogs in run 8. The results of these analyses are summarized in Table XII.

The average control and experimental P/O ratios were 2.78 and 3.13, respectively. They are not significantly different and are similar to normal values for rat liver. All the NAD/NADH ratios ranged from 5.5 to 6.2. This relatively narrow range of values shows no significant oxygen effect. The values, however, are lower than the acceptable range for rat liver, namely 8 to 10.

Table XII. Liver mitochondrial P/O ratios and liver NAD/NADH ratios for monkeys exposed in the Thomas dome to oxygen at 258 mm Hg.

Run No.	Atmosphere; Time	P/O	NAD/NADH
9	Air control; 236 days	3.23, 2.92	5.5
		2.43, 2.55	5.8
		Avg. 2.78	Avg. 5.65
	Oxygen; 236 days	3.67, 3.28	6.2
		2.78, 2.78	5.9
		Avg. 3.13	Avg. 6.05
	Oxygen; 236 days + air; 40 days	3.74, 3.43	5.1
		3.91	4.9
		Avg. 3.78	Avg. 5.0
11	Oxygen; 72 hr	2.39, 2.34	20.9
		*	5.3
		Avg. 2.37	

\* Insufficient sample was available.

Separate monkeys were allowed to recover in air for 40 days after oxygen exposure. The average P/O ratio after recovery was 3.7, slightly higher than the control or oxygen values. The NAD/NADH ratio, averaging 5.0, was slightly lower. These changes are not considered significant; more experimental animals and simultaneous recovery controls are required.

In run 11, two monkeys were exposed to pure oxygen at 258 mm Hg pressure in the Thomas dome for 72 hr. Analyses for liver mitochondrial P/O ratio and liver NAD/NADH ratio were carried out. The P/O ratios were similar to those for run 9; the NAD/NADH values ranged from 5.3 to 20.9.

## SECTION IV

### DISCUSSION

#### A. Significant Biochemical Indexes of Oxygen Toxicity

A sensitive biochemical index of oxygen toxicity that appears early enough during exposure to enable complete protection of the individual from irreversible damage upon removal from the environment would be an important advance in the use of artificial oxygen atmospheres. Such a finding might be comparable to the clinical use of serum isoenzyme levels and distribution patterns for pinpointing damage to specific tissues. Even without comparable specificity, with sufficient sensitivity and with appearance early during exposure, such an index would have great utility when oxygen constitutes the only known hazard; for example, the nature of the biochemical aberration might suggest suitable protective measures that could be taken.

A distinction between the statistical significance and the biochemical significance of a particular experimental finding must be made. With suitable experimental design and use of sufficient numbers of animals, such as rats, pertinent statistical computations enable us to determine the extent of the nonrandom oxygen effects. Furthermore, at a given cost much more information can be obtained from a symmetrical multidimensional set of statistics that permits cross-correlations than from merely a linear array of data.

In the absence of large numbers of animals, internal controls, a larger variety of related biochemical analyses, and more replicate experiments are used. Thus monkeys have been subjected to repetitive biopsy for internal control in morphological studies. The objective of any experimental design and statistical analysis is to establish with reasonable confidence that the results can be related to specific parameters.

The biochemical significance of the particular effect in relation to oxygen may be more difficult to establish. Questions that must be answered include:

- (1) Is the effect caused by, rather than merely related to, oxygen exposure?
- (2) Is the effect specifically caused by oxygen or can other agents produce the same response?

- (3) Is the response directly due to oxygen or is it an indirect measure of some other target effect?
- (4) Is the response the major one produced by oxygen?
- (5) Are we measuring the response at the most refined organizational level, i.e., subcellular, mitochondrial, molecular?

Providing these answers may enable the biochemist to unravel the metabolic reaction mechanisms that are directly involved in oxygen toxicity. Findings in related areas, particularly visualization of ultrastructures by electron microscopy and use of histochemical techniques may be applicable toward the final solution. The objective is to gain sufficient understanding at a subcellular level to establish a rational basis for formulating protective measures.

The two indices that appeared most significant upon exposure of rats to oxygen at 1 atm, namely, weight loss and uncoupling of oxidative phosphorylation, are excellent examples of relatively indirect and direct responses at a physiological level. Weight loss is a reflection of the balance of a host of anabolic and catabolic reactions in the animal body. It may, however, merely reflect the decreased appetite of the animal that is intoxicated by oxygen. The decreased appetite could in turn be related to general nausea or malaise, which is a part of this syndrome. Weight loss due to anorexia is associated with many human illnesses. There can be no question that the animals are morbidly ill. Undoubtedly, a key physiological structure or biochemical metabolic pathway is impaired by hyperoxia.

The uncoupling of oxidative phosphorylation is not necessarily a demonstration of a direct effect of oxygen but it is a response that is considerably better defined and operates at a microscopic morphological level within the cell. First, the effect is demonstrated with fractionated mitochondria from tissues of exposed animals. Moreover, we know that most of the transfer of oxidative energy to phosphorylation occurs along the electron transport chain on the route to molecular oxygen. Recent studies show that electron transport is associated with particles adjacent to the cristae within the mitochondrion. Hence, even if oxygen modifies this energy transfer indirectly by some shuttle mechanism from outside the mitochondrion, the effect is far more amenable to biochemical analysis than weight change.

The importance of this finding is that nearly all the energy in cell function is derived directly from high-energy phosphate produced in this manner. Any impairment of the efficiency of this process is met by dire consequences.

Increased power in the use of biochemical or physiological indexes for detection comes from mathematically lumping them into one composite effect. Not only does such a composite index offer greater sensitivity, but its coefficients can suggest altered patterns of the toxic syndrome. The greater sensitivity offered may be required for the detection of smaller, less acute responses with exposure at 1/3 instead of 1 atm. Thus, only subtle changes in P/O ratio may be detectable at 1/3 atm, while the significance of weight change is five times that of P/O ratio at 1 atm.

A further use of the composite index is the comparison of the response obtained with rats exposed to oxygen at 1 atm in either the Felig-Lee chamber (runs 5 and 6) or the Thomas dome (runs 3 and 4). Examination of the two indices that make up the composite shows a similar reduction of weight gain in experiments in both chambers. However, the decrease in P/O ratio was considerably greater in the Felig-Lee chamber than in the Thomas dome.

Certainly additional individual indices sensitive to oxygen exposure will greatly increase the versatility and power of a composite index approach to the detection of oxygen intoxication.

#### B. Relation to Proposed Biochemical Mechanisms of Oxygen Toxicity

The history of investigations in the field of oxygen toxicity is long and diversified. In evaluating the significance of the findings to the present problem, several guidelines should be emphasized. First, the numerous experiments with in vitro enzyme systems, many of which have shown oxygen sensitivity, merely show the range of response possible in the highly denuded state. We contend that experiments in which whole animals are exposed to oxygen will be more likely to yield pertinent information. The extremely complex systems of cellular oxygen delivery, metabolic control by feedback and shuttle mechanisms, and intact cellular morphology are absent in an in vitro soluble enzyme system. Fractionated intact mitochondria, though removed from the cell, reveal the functional integrity of energy utilization mechanisms that certainly operate within the cell subject to extramitochondrial control.

Second, it appears that man evolved on this planet with antioxidant defense mechanisms for combating destructive effects of oxygen upon living tissue (ref. 18). In a real sense, he tolerates oxygen as a double-edged sword that supplies energy for life with the ever-present possibility of oxygen toxicity. The range of oxygen concentrations that particular individuals can tolerate under special circumstances may be now narrower than is generally recognized. Parameters that can accentuate oxygen toxicity included fatigue, presence of atmospheric contaminants, infections, prolonged exposure, and individual variation in susceptibility.

Finally, oxygen probably has numerous biochemical effects. The important search is for the target that, at molecular and tissue ultrastructural levels, becomes limiting in a particular set of experimental circumstances. Thus under hyperbaric conditions, death can ensue from immediate central effects leading to convulsions. When these effects are controlled, death can ensue from other toxic manifestations of oxygen toxicity. If the lung is damaged by early hyperoxic exposure, the impairment of gaseous exchange and resistance to airborne toxic materials and infectious agents can predominate. If the erythrocyte membranes are damaged, hemoglobin loss can become critical. If no other acute tissue damage is demonstrated, the effect of oxygen in energy utilization within cellular mitochondria becomes basically predominant. Certainly, the patterns of oxygen toxicity with prolonged exposure at low oxygen concentrations may be quite different from those used in hyperbaric therapy.

Two oxygen toxicity mechanisms that have been proposed should be mentioned. The first is lipid peroxidation (ref. 19). The role of lipids in maintaining the integrity of cell membranes is well known. Oxygen has been shown to destroy artificial lipid bilayer membranes (ref. 20). Apparently, the development of antioxidant mechanisms is necessary for the maintenance of biological membranes in the presence of an oxygen atmosphere. Lipids are essential to the integrity of the central nervous system, and conceivably some of the central effects of hyperbaric oxygen exposure may be due to oxidative lipid reactions. The mitochondrion contains 25 to 30% phospholipids. The lipid fraction can be extracted from the mitochondrion without altered appearance of the structure by electron microscopy. Furthermore, electron transport activity is restored to the mitochondrion by the addition of lipoidal materials. In this case, the lipid fraction acts as a kind of solvent or matrix in which electron transport mechanisms function.

The second proposed mechanism of oxygen toxicity involves the ox-redox state of the pyridine nucleotides. In their studies at 6570th AMRL, Felig and Lee (ref. 4) suggested that the availability of NADH is crucial in the tolerance of excess oxygen. Various buffering agents were administered to rats intraperitoneally during exposure to pure oxygen at 1 atm. Lactate increased the survival rate, but TRIS, bicarbonate, acetate, and pyruvate did not. It is of interest that among these substrates lactate uniquely generates NADH. Felig and Lee postulated that exposure to oxygen at increased concentration may result in depletion of NADH that is restored by lactate. The role of other NAD-coupled metabolites is of interest in this connection.

Sanders (ref. 21) found that succinate protects against hyperbaric oxygen toxicity. Succinate is believed to function by maintaining necessary energy stores (ATP levels) in tissues. These levels are drastically reduced in hyperbaric oxygen toxicity without succinate protection. Since succinate transfers hydrogen to flavin rather than pyridine coenzymes, it presumably bypasses the NAD/NADH system.

Administration of succinate to protect against oxygen toxicity for prolonged periods of time might render the pyridine nucleotide systems nonfunctional. Both NAD and NADP are required in many essential metabolic reactions. Furthermore, whether lactate exerts its action by supplying NADH can also be questioned. Lactate probably does not enter the mitochondrion directly. Further, NADH produced anaerobically also most likely cannot enter the mitochondrion to function in aerobic oxidative metabolism. It is more likely that lactate functions in a more remote capacity in extramitochondrial control mechanisms.

No discussion of the biochemistry of oxygen toxicity could be complete without reference to the classic findings by Chance and coworkers (ref. 17). By using direct fluorometric techniques with a variety of biological preparations, including whole animals with exposed organs, isolated mitochondria, submitochondrial particles, and other cellular species, an universal sensitivity of the pyridine nucleotide system to oxygen was shown. The general finding is that low oxygen concentration causes increased NADH and that high oxygen concentration causes disappearance of the reduced coenzyme. The response was rapid, i.e., within minutes, and could be repeated through 4 to 6 cycles. It is of interest that intact mitochondria are required to show this response in an in vitro system.

The theoretical aspects of this phenomenon are exceedingly complex. The reversed electron transport chain appears to be involved. One conclusion is that hyperbaric oxygen inhibits the use of high-energy intermediates in energy-linked NAD reduction.

The lack of any significant change in NAD/NADH ratio in animals exposed to either 1 atm or 1/3 atm of pure oxygen in our studies may reflect the capacity to adapt to oxygen concentrations lower than hyperbaric. It should be pointed out that the measurements of Chance were made during or immediately after exposure to hyperbaric oxygen for much shorter periods of time.

Final reference is made to morphological studies that have been conducted by several investigators in related programs at 6570th AMRL. Histopathological studies by Robinson (ref. 22) and electron micrographic and morphometric studies by Felig (ref. 23) and Kistler (ref. 24) have shown dramatic changes in lung as a result of exposure to pure oxygen at 1 atm. There can be little question that the gas exchange across capillaries in the edematous state is severely impaired. Of particular interest are the findings of Schaffner (ref. 25) in liver mitochondria of rats exposed to pure oxygen at 1/3 atm by electron microscopy. A typical sequence of events is as follows:

- (1) Loss of glycogen, 3 days
- (2) Enlarged mitochondria with pinching at the waist, 1 week
- (3) Increased numbers of mitochondria, 2 weeks
- (4) Increased numbers of cristae
- (5) Clustering of mitochondria
- (6) Increased numbers of polyribosomes
- (7) Glycogen back to normal, 90 days
- (8) Larger mitochondria with internal crystalline material, 8 months.



Although the exact sequence varies with different species of animals, two types of changes are evident: the early damages, including glycogen depletion and enlargement of mitochondria, and subsequent increase in polyribosomes, suggesting increased protein synthesis.

The changes found at 1 atm included mitochondrial enlargement, cristae loss and mitochondrial dissolution within 1 or 2 days; some changes occurred at 6 hr.

Most of the evidence presently indicates that the major and most fundamental effect of excess oxygen is the derangement of mitochondrial structure and function. That changes in oxidative phosphorylation at the biochemical level and in the cristae at the morphological level should both be found in approximately the same time sequence in the liver cells of animals exposed either to 1 atm or 1/3 atm of pure oxygen amply demonstrates the utility of both approaches. The entire history of mitochondrial research shows that real progress was not made until a happy marriage between structure and function had been accomplished.

### C. Selection of Analytical Procedures

Detection of any physiological or biochemical functional impairment due to oxygen exposure is predicated upon our ability to select and carry out suitable analyses. The selection of pertinent analyses for these studies was based to a considerable degree upon the findings of previous workers, including Felig at 6570th AMRL, in this field. The finding that oxidative phosphorylation in rats is partially uncoupled by exposure to 1 atm of pure oxygen attests to at least limited success in extrapolation from previous experimental findings.

In addition, not only have we attempted to select those biochemical analyses that span crucial metabolic sequences in vivo, but also our selection has been enlarged by a somewhat intuitive sense. Thus, although our prime objective was to find sophisticated biochemical evidence of oxygen toxicity, the importance of negative animal weight changes were in a sense an afterthought, even though careful records of animal weight were routinely kept in all experiments. The point is that this index was the most powerful for these rats at 1 atm and yet was the most easily carried out.

To gain an understanding of biochemical mechanisms, however, more elaborate analytical methods are required. The desirability of seeking evidence at several levels of tissue and cellular organization is generally accepted. For mechanisms at an in vivo cellular and subcellular level, the use of whole animals cannot be avoided. In this case, changes during oxygen exposure are monitored, tissue bits are removed by biopsy during exposure, or the animal is sacrificed after exposure for specific tissue analyses.

The first of these is very limited in applicability. The classic use of this approach is that of Chance (ref. 17) in which NAD sensitivity to wide ranges of oxygen concentration was shown in vivo by direct monitoring with telescopic fluorescence techniques. This work utilized both intact animals with exposed organs and mitochondrial preparations in vitro. The second technique, the biopsy method, was used widely in our experiments. It has the advantage of internal control in a given animal, although the imposed stress due to repeated biopsy must be considered. This method is limited by the number of organs that can be biopsied.

The third method, namely, animal sacrifice after exposure, was most widely used in these experiments. The major problem of this approach is the transition from the treated animal to the biochemical analysis. The sequence of chamber animal removal to an ambient air environment, anaesthesia, animal surgery, tissue removal, and tissue processing can cause a host of artifacts. The problem is particularly acute in the NAD/NADH analyses. We have attempted to overcome this by using in situ tissue freezing. In some preliminary experiments we have removed tissues for this analysis by direct biopsy or in situ freezing while the animal was in the chamber. Further work is required to perfect these techniques.

Finally, we cannot overemphasize selection of the analytical method that has the greatest sensitivity to changes in the metabolic state of the animal. Thus the older methods for analysis of NAD/NADH, which did not utilize tissue freezing, enzyme cycling, and fluorometry, may have completely missed ox-redox changes if they occurred. Furthermore, methods more sensitive than P/O ratio for measuring uncoupling of oxidative phosphorylation are available. Another example is the use of serum biochemical changes to reflect acute cellular and subcellular damage. Specific lesions that occur in the mitochondrion

most likely will not be reflected in the blood during the initial stages of the lesion. It is of interest that no significant changes have been found in animals exposed at 1/3 atm by the use of numerous clinical blood analyses (ref. 26).

#### D. Comparison of Animal Species and Environmental Conditions

The selection of suitable animal species for the study of oxygen toxicity has a considerable bearing upon the kind of results that will be obtained. First, to study the biochemistry of oxygen toxicity, we need to select animals that will show evidence of susceptibility to such toxicity. In one chamber or another, some strains of rats will not show evidence of toxicity to pure oxygen even at 1 atm of pressure. In the work described here, we have concentrated on the repeated use of conditions that show an oxygen sensitivity. The precise cause of increased oxygen sensitivity is not necessarily known -- it may be a complex package that includes a specific animal strain, a particular environmental chamber, and other factors. We believe that this fact alone shows that little is known about the interactions of sensitizing factors. The use of composite biochemical indexes such as described previously is particularly pertinent.

If these studies are ever to be applied to the problem of manned space flight in pure oxygen atmospheres, the use of several animal species and chamber environments becomes extremely important. Perhaps only one human astronaut will, for an unknown reason, show oxygen sensitivity similar to that shown by a susceptible animal. Such a finding would not be new in clinical medicine. Certainly the systematic approach is to use large numbers of animals that have susceptibility and genetic uniformity for some experiments and to use limited numbers of larger animals, including several kinds of primates, for other experiments.

### E. Adaptation, Recovery, and Protection

The experiments described have shown evidence both of adaptation and of recovery. In discussing these phenomena, note that the objective in experiments with oxygen at 1 atm is to gain an understanding of mechanisms rather than simulation of space-flight conditions. In runs 1 and 2, evidence of recovery in air from the adverse biochemical effects of exposure to oxygen was obtained. In fact, all three indices, namely, weight change, P/O ratio, and liver mitochondrial LDH, showed the extent of this recovery. Although this finding is preliminary, it is startling: if the animals remained in oxygen a few days longer than 72 hr, they would have died. There can be no question that, up to a point, even adverse reaction to oxygen is reversible on subsequent exposure to air. We believe that this phenomenon including various cycles of oxygen and air exposure, should be much more intensively investigated.

The metabolic picture at 1/3 atm of oxygen may be quite different. Early biochemical changes may cause alarm unless it is realized that they may represent adaptation by the animal. Such changes may be temporary -- i.e., they may return to normal values with prolonged exposure. This was the case in our slightly elevated P/O ratios in the first week or two in rats exposed to oxygen at 1/3 atm. In fact, in this case we could not be sure what the beneficial effect of a temporary "tightening up" of oxidative phosphorylation may be. On the other hand, the early changes may not ever return to the same levels found in animals breathing air. Such a case does not necessarily denote unsuccessful adaptation. A new oxygen environment may require a new set of biochemical mechanisms.

In the event that the animal, or a particular astronaut, does not successfully adapt to a pure oxygen atmosphere, the administration of protective substances has considerable importance. However, such substances may not be easily found. Thus the administration of succinate, simply because it has been found to protect against hyperbaric oxygen toxicity in animals (ref. 21), may not be suitable as a protective agent in manned space flight. This substrate, if used as a major energy source, may bypass the pyridine nucleotides that are essential in a host of biochemical reactions. The integrity of the erythrocyte membrane depends, for example, upon proper

functioning of pyridine nucleotides. It is well known that enzyme syntheses can be substrate-induced. Hence changing from a pyridine nucleotide-dependent pathway to the flavin route can have adverse consequence.

An additional problem is access to the presumed site of oxygen sensitivity. Even if several mechanisms of oxygen toxicity are apparent, the major site of impairment seems to be in the mitochondrion. Many substances, including the pyridine nucleotides, will not enter a mitochondrion with an intact membrane. Hence, the parenteral injection of any substance must either enter the mitochondrion or affect electron transport pathways within the mitochondrion remotely by some shuttle mechanism. We believe that the study of these shuttle mechanisms as affected by hyperbaric oxygen is appropriate. The only investigator that has made real progress in this field is Chance (ref. 17).

The approach is a difficult one, but it may greatly increase our understanding of one of the most pressing problems in human alimentation. That the very oxygen we breathe could in itself impair the transfer of hydrogen from organic substrates to oxygen to form water and generate high-energy phosphate bonds is a surprising feedback mechanism. The desire of man to alter his atmospheric environment for a host of ventures makes it imperative that he gain a real understanding of the effects such manipulation will have upon his own well-being.

## SECTION V

### CONCLUSIONS

For the preliminary phase of this program, in which analytical methods were developed and normal values for rats were obtained, we have reached the following conclusions.

1. Fractionation of tissue mitochondria that respire uniformly with NAD-linked substrates at maximal rates requires strict adherence to procedural details. This fractionation was accomplished with liver but not with lung tissue. The liver mitochondrial  $Q(O/N)$  rates were highly reproducible; in general, they ranged from 85 to 95  $\mu$ liters/10 min. The P/O ratios were acceptable, averaging 2.89 (as compared with the value of 3.2 obtained by Lardy), and also were in a relatively narrow range.  $Q(O/N)$  rates for lung were more variable than those for liver. P/O values were not calculated since phosphorylation was not accomplished with our preparations. Hence lung mitochondria present unusual difficulties that require further investigation.

2. Liver NAD values (608) were similar to those obtained by Bruch and Dippe (700), and NADH values were approximately 50% higher (78 versus 50)\*. NAD/NADH ratios had a rather wide range but averaged from 8 to 9 in comparison with 10 to 20 obtained by Burch and Dippe. Our values for the ratio do agree with those currently being obtained by Chance and by Lardy (see Appendix V).

3. Arterial blood lactate values approximate the range obtained by other investigators. However, the pyruvate values obtained in our work were somewhat lower. These lower values could be due to a greater specificity in our method of analysis. The ratio is affected accordingly.

4. In general, animal-to-animal variation was much greater than tissue-to-tissue variation from the same animal. Since run-to-run variation was often comparable to animal variation, groups of animals were used and repetitive runs were made.

For experiments in which rats were exposed to pure oxygen at 1 atm in the Felig-Lee chamber and in the Thomas dome for periods up to 96 hr we have reached the following conclusions.

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\* Micromoles/kg.

1. Liver mitochondrial Q(O/N) was not significantly affected by exposure to oxygen.

2. Liver mitochondrial P/O ratios were significantly decreased ( $P = 0.05$ ) during oxygen exposure for 72 to 96 hr by mean amounts that ranged from 0.06 to 0.95 units. Although considerable run-to-run variation was found, statistical tests for residual error showed significance with respect to either a time or an oxygen effect ( $P = 0.01$ ). Analysis of these data showed that simultaneous air controls were required for each period of oxygen exposure. Nearly complete recovery was found after subsequent exposure to ambient air for 3 to 10 days. Although few animals were used in this experiment, the recovery is striking. Hence, although oxygen exposure can be lethal to the animal and can seriously interfere with the efficiency of oxidative phosphorylation, the impairment is reversible after 3 days of exposure.

3. Lung mitochondrial Q(O/N) was not significantly affected by oxygen exposure. However, the use of NAD- rather than flavin-linked substrates could modify this finding.

4. Liver mitochondrial LDH values showed a significant increase ( $P = 0.05$ ) during exposure to oxygen for 48 hr. The individual results varied widely from run-to-run. Data reduction to eliminate the run-to-run variation was required to show the effect. Reversal of the values occurred after subsequent exposure to air.

The liver LDH isoenzyme distribution was not significantly affected by animal oxygen exposure. The mitochondrial isoenzyme was predominantly the anaerobic cathodic band 5; homogenates showed significant amounts of the aerobic bands.

5. In general, liver and lung NAD, NADH, and NAD/NADH did not show significant changes with exposure to oxygen. This is of interest because NADH is very sensitive to changes in the anaerobic state of tissue. More refined methods may show an altered NADH level as a result of oxygen exposure.

6. Blood lactate and pyruvate both increased as a result of oxygen exposure; however, no significance in the resulting ratios was noted. This result suggests an increased rate of general glycolysis. However, it is doubtful that the mitochondrial LDH changes can account for these changes in the

blood levels of LDH reaction products, namely, lactate and pyruvate. Apparently, mitochondrial LDH reflects anaerobiosis as a result of exposure to oxygen at 1 atm. The increase in LDH activity is most likely a reflection of increased enzyme synthesis. The biochemical role of mitochondrial LDH was not clarified.

7. Animal weight was significantly changed ( $P = 0.01$ ) by oxygen exposure. The change after 96 hr of exposure was -25.4%. Reduction in food consumption was observed in the oxygen-exposed animals. Hence the decrease in weight gain is probably largely due to anorexia.

8. The significant oxygen and time effects are summarized below (X = significant; n.s. = not significant).

Index	Univariant Analysis		Multivariant Analysis
	Oxygen Effect	Oxygen X Time	
Liver mitochondrial Q(O/N)	n.s.	n.s.	n.s.
Liver mitochondrial P/O	x	x	x
Liver mitochondrial LDH	x	x	n.s.
Lung mitochondrial LDH	n.s.	n.s.	n.s.
Liver NAD	x	n.s.	-
Liver NADH	n.s.	n.s.	-
Liver NAD/NADH	n.s.	n.s.	-
Blood lactate	x	n.s.	-
Blood pyruvate	x	n.s.	-
Blood lactate/pyruvate	n.s.	n.s.	-
Weight loss	x	x	x



9. A composite index consisting of a linear regression equation can be derived from the multivariant analysis:

$$\text{Index} = \log W_2/W_1 + 1/5 \log P/O$$

where  $W_1$  and  $W_2$  are initial and final animal weights, respectively, and  $P/O_1$  is the  $P/O$  ratio.

Application of this index to data from animals exposed to oxygen under different conditions, i.e., different chambers, showed different patterns of response to oxygen.

For experiments in which rats were exposed to pure oxygen at 1/3 atm in the Thomas dome for periods up to 236 days, we have reached the following conclusion.

1. Exposure for 3 to 7 days showed a rise in the mean  $P/O$  ratio, which resumed a normal level after 14 and 236 days. The significance of this effect was not determined and will require further investigation. The inference is that animals show an initial "beneficial" tightening of oxidative phosphorylation at this oxygen concentration and subsequently adapt to the new environment with a return to normal efficiency levels. No significant weight changes were observed at this pressure. Hence the pattern of the response was considerably different from that found at 1 atm.

Experiments with monkeys at 1 atm in the Thomas dome for periods up to 170 hr, dogs at 1/3 atm in the Thomas dome for periods up to 236 days, and monkeys at 1/3 atm in the Thomas dome for periods up to 236 days (including a 40-day air recovery) have been described. These experiments were preliminary; hence no conclusions can as yet be made. There is an indication that monkeys are more resistant to oxygen toxicity at 1 atm than rats.

## APPENDIX I

### ANALYTICAL METHODS

#### 1. Preparation of Mitochondria

The lability of cellular mitochondria has been well established. Attempts to isolate these subparticles from their native cellular environment while retaining their unique respiratory and oxidative phosphorylating capabilities have often been frustrated. Successful separation and isolation of intact, viable mitochondria has been accomplished by using synthetic media that correspond to the natural cytoplasmic medium of the living cell. Sufficient regard for such limiting requirements as osmolarity and pH of the medium and its essential ionic composition (sodium, potassium, magnesium, and manganese) has been cultivated mainly through trial and error procedures. The morphological and enzymatic quality of mitochondria isolated by any technique, no matter how sophisticated, can approach that exhibited by the mitochondria in its natural cellular environment, but the quality has been sufficient enough to permit extensive study of the mitochondrial ultrastructure and many aspects of oxidative reactions. This study was carried out by using a fractionation method described by Lardy (ref. 7).

Livers and lungs were excised from rats that had been decapitated and desanguinated, and the organs placed immediately into ice cold isotonic sucrose solution (0.25 M sucrose, A.R., in triple distilled water and containing TRIS,  $3.2 \times 10^{-4}$  mole/liter, total solution adjusted to pH 7.4 with 5 M potassium hydroxide).

The livers and lungs were weighed in the cold sucrose solution by differential weighing on a Harvard trip balance (sensitivity 0.1 g). To each gram of tissue, 8.1 ml of ice cold sucrose solution was added and the mixture thoroughly homogenized by using a ground glass flask and conical pestle (Kontes glassware) drive by a variable-speed stirring motor (E. H. Sargent). During homogenization the flask and its contents were kept cold by constant immersion in an ice bath. The homogenate was transferred to either 15- or 50-ml lusteroid tubes (International centrifuge) and the nuclear fractionation carried out by centrifuging for 10 min at  $600 \times g$  in a refrigerated International centrifuge (model HR-1) maintained at 0 to 4°C.

Then the supernatants were carefully decanted into another series of tubes and reserved in an ice bath for the mitochondrial fractionation. The remaining pellets were slurried with a large-diameter (8 to 10 mm) glass rod and then resuspended in 0.9 ml of the cold sucrose solution per g of initial liver tissue. This washing step is essential for reclaiming the large mitochondria that sedimented during the nuclear centrifugation. The tubes were again spun under the same conditions and the supernatants combined with those from the first spin. This pool in turn was centrifuged at 17,500 x g for 5 min in the cold (mitochondrial fractionation). The supernatants from this spin containing the cytoplasm, and ribosomes and fluffy layer (lipid layer) were discarded. The mitochondria appeared as a heart-shaped pellet at the bottom of the tube. The bulk of the pellet was dark beige, surrounded by an edge of pink material. The mitochondria were washed twice by resuspending in ice cold sucrose (1.5 ml per g of initial tissue) centrifuging at the same high speed. The supernatants containing the pink material were decanted after each spin. The mitochondrial pellet was resuspended in cold sucrose, 1 ml/g of starting tissue, by homogenizing it in a small glass homogenizing unit, and this preparation was kept at ice bath temperature. Mitochondrial respiration studies were made by using a Warburg method described by Lardy (ref. 7). For liver preparations a system containing alpha-ketoglutarate was used and for lung mitochondria succinate was used. The reaction mixture contained in each Warburg flask is detailed below:

<u>Flask</u>	<u>Liver</u>	<u>Lung</u>
Sucrose	0.25 M	0.25 M
Potassium phosphate	0.1 M	0.1 M
Potassium alpha-ketoglutarate	0.3 M	-
Sodium succinate	-	0.3 M
Disodium adenosine triphosphate	0.1 M	0.1 M
Magnesium chloride	0.15 M	0.15 M
Liver mitochondrial suspension	Yes	-
Lung mitochondrial suspension	-	Yes

#### Side Arm

Glucose (0.25)M + hexokinase (1.3 g/ml)	Yes	Yes
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#### Center Well

Potassium hydroxide	5.0 M	5.0 M
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## 2. Liver Mitochondrial Respiration

For the liver respiration study the above reagents less the mitochondrial suspension were carefully added to each single-arm Warburg flask (15 ml capacity) and cooled to 0°C in an ice bath. Mitochondrial suspension was added to duplicate flasks for the respiratory study and to a single flask for the zero time study. All joint surfaces were coated thinly with pure hydrolyzed lanolin (Merck). Each flask was carefully attached to a matched double-capillary manometer and placed into the Warburg bath (Precision Instruments), which had been equilibrated to 30°C. The remaining flasks were inserted at 30-sec intervals and allowed to equilibrate 8 min before the manometer valve was closed. After 2 min of additional equilibration, the initial manometric readings were taken and the contents of each flask thoroughly mixed by careful tipping.

The flasks were replaced in the water bath and the reaction carried out for 10 min, at which time the final manometric reading was taken, the flask removed, and 2 ml of 10% trichloroacetic acid added. The acidified mixture was kept cold for 15 min to allow complete precipitation of all protein constituents and finally filtered through paper (Whatman No. 1).

The zero time flask was treated in the same manner as the 10-min flask; however, the reaction was terminated immediately after the contents were mixed. The differential manometer readings recorded as millimeters of pressure were corrected for thermal barometric changes and converted to a volume relationship by using the appropriate flask and manometer constants. This volume represents the  $\mu$ liters of oxygen consumed during the respiration study, which in turn were converted to atomic equivalents.

The degree of oxidative phosphorylation that occurred during any mitochondrial study was reflected in the disappearance of inorganic phosphate. The amount of inorganic phosphate converted to ATP was determined differentially from the zero time and 10-min flasks by employing the Fiske-Subarrow method (ref. 27) without modification. The differential values obtained were expressed as microatoms of phosphate converted to ATP.

Q(O/N) for liver mitochondria was determined by using the following expression:

$$Q(O/N) = \frac{\mu\text{liter of oxygen consumed per 10 min}}{\text{mg of mitochondrial nitrogen in flask}}$$

The phosphorous to oxygen ratio (P/O) was calculated from the following expression:

$$P/O = \frac{\text{microatoms phosphate converted to ATP}}{\text{microatoms oxygen consumed in 10 min}}$$

### 3. Lung Mitochondrial Respiration

Since the lung mitochondria isolated by the above ultra-centrifugal method did not retain their oxidative phosphorylating capacity, only Q(O) measurements were made. These measurements were carried out in a manner analogous to that of the liver mitochondria; however, the respiration were continued for 60 min instead of the 10-min period normally used for liver mitochondrial respiration. The Q(O/N) values for lung mitochondria expressed in this report were determined from the following expression:

$$Q(O/N) = \frac{\mu\text{liter oxygen consumed per hr}}{\text{mg of mitochondrial nitrogen in flask}}$$

### 4. Lactic Dehydrogenase Isoenzymes

In order to investigate the effect of pure oxygen environments at 1 atm and at 1/3 atm of pressure upon the isoenzyme constituents of LDH in liver and lung mitochondria, samples of mitochondrial suspension from each organ were analyzed electrophoretically in an agar gel medium.

For this purpose gel slides were prepared from an 0.8% suspension of purified Nobel agar in veronal buffer, pH 8.6, ionic strength 0.05. The gel was layered on a supporting glass microscope slide to a thickness of approximately 1 to 2 mm. Aliquots of 5- $\mu$ liter of mitochondrial suspension were applied to the narrow trough cut in the center of the gel slide. The material was allowed to absorb in the gel, and the slide placed across the agar electrodes of a modified Wieme electrophoresis cell. Electrophoresis was carried out for 30 min by using a constant current

of 30 mamp/slide and a potential gradient of approximately 130 volts. The cell compartment containing the electrodes and gel slides was filled with petroleum ether (20 to 40°C) to reduce the temperature due to ohmic heating.

After electrophoresis was complete, the slides were transferred to petri dishes, an incubation mixture added, and the slides allowed to develop for 1.5 hr at 37°C. The incubation mixture contained the following:

Sodium cyanide	0.1 M
Nitroblue tetrazoleum	5 mg/ml
Sodium lactate syrup	60%
Sodium phosphate buffer, pH 7.1	0.1 M
NAD	6.66 mg/slide
Phenazine Methosulfate/mg/ml	

The slide was developed by the deposition of reduced nitroblue tetrazoleum in the areas of LDH isoenzymes, thus forming discrete blue bands. Excess reagent was eluted out of the slide and the isoenzymes fixed in methanol:acetic acid:water (45:5:45). The slides were then dried in the dark and the isoenzyme band intensities measured with a Beckman/Spinco model RB Analytrol with a neutral density filter.

## 5. LDH Activity

Lactic dehydrogenase activity was quantitatively determined by using the method of Berger and Broida (ref. 11). The measurement was performed on the mitochondrial suspensions used to obtain P/O ratios on the Warburg apparatus. These mitochondrial suspensions were diluted 1:50 (0.1 ml suspension added to 4.9 ml water). Since the Berger-Broida dilution is 1 part serum to 5 parts water (1:6) and this dilution is 1:50, the Berger-Broida units (BB) obtained in the analyses must be multiplied by 8.33. LDH activity was reported as BB units/mg N. Frozen samples were used for the analyses. It is of interest to note that samples frozen for 6 months retained their original activity.

## 6. NAD and NADH Determinations

Oxidized and reduced NAD values were determined on liver and lung tissues by enzymatic cycling. The method of Lowry et al. (ref. 9) was modified to incorporate speed and to facilitate use of the Beckman DU spectrophotometer. Liquid Freon was obtained by passing bottled Freon gas through coils immersed in liquid nitrogen. The Freon was stored on dry ice.

Birch and Von Dippe (ref. 8) first described the method of freezing liver in situ. They found by freezing in situ the NADH values were considerably reduced. Other investigators decapitated the animals prior to NAD and NADH analyses. It was postulated that the ischemic (hypoxic) condition affected by decapitation caused elevated NADH values. Subsequent analyses affirmed this supposition.

We decided therefore to analyze NAD and NADH via in situ freezing. Modified polypropylene disposable beakers provided a convenient dish for freezing. Polypropylene does not adhere to the surrounding tissues upon freezing. Liver and lung tissue can thus be easily isolated and severed.

After light ether anaesthesia the liver was exposed, placed in the dish (with the aid of a hemostat), and immediately frozen. Care was taken to prevent rupture or damage to the liver while freezing. The liver was immersed until lung removal.

By cutting the rib cage bilaterally, the entire thoracic cavity was exposed and the left lung frozen. Both freezing processes required approximately 1 min. Samples of tissue (100 to 200 mg) were weighed on a Roller Smith precision balance. Two controls and three experimental lung and liver tissues were used each day. Few samples were stored.

For NAD measurements, liver samples were diluted 1:50 and homogenized in 0.01 M sulfuric acid and 0.1 M sodium sulfate. Lung samples were homogenized by using a 1:25 dilution. For NADH measurements, liver samples were diluted 1:50 and homogenized in 0.02 N sodium hydroxide. Lung samples were homogenized by using a 1:25 dilution. For NADH measurements, liver samples were diluted 1:50 and homogenized in 0.02 N sodium hydroxide. Lung samples were homogenized by using a 1:25 dilution. Prior to NADH homogenization, 8 mg cysteine hydrochloric acid was added to 100 ml of the 0.02 N sodium hydroxide. Both liver and lung samples were homogenized by utilizing a Kel-F apparatus. A Sargent cone drive stirring motor provided suitable power.

NAD samples were incubated 45 min at 60°C and NADH for 10 min. After incubation, the samples were centrifuged for 10 min at 4300 to 4800 rpm. All liver samples were further diluted 1:5.

The cycling mixture was made in 111-ml quantities, of which 10 ml aliquots were frozen. This mixture was prepared as follows: 0.2 M TRIS buffer (2.688 g), 0.03 M sodium ADP (16.2 mg), 100 micromoles sodium lactate dissolved in 16.82 ml of 0.66 N sodium hydroxide, 5 micromoles alpha-ketoglutaric acid (101.6 mg). The mixture was adjusted to pH 8.4.

A test tube rack containing 12 x 75-mm test tubes was kept in a pan of ice water; 0.05-ml samples or standards\* were pipetted into the tubes. To each tube was added 0.2 ml of cycling mixture. Prior to this addition, 0.2 ml of glutamic dehydrogenase (5 mg protein) and 0.050 ml beef heart lactic acid dehydrogenase (0.5 mg protein) was included in the mix. The tubes were incubated 30 min at 30°C. After incubation, the tubes were placed in boiling water for 2 to 2.5 min.

To each tube was added 2.5 ml of phosphate buffer. The buffer contained 0.65 M sodium monobasic phosphate and 0.15 M potassium dibasic phosphate adjusted to pH 6.3. Just prior to the addition of the buffer, 10 mg NADH per 40 ml buffer was included. The contents were stirred, read at 340 mμ, and the optical density (O.D.) recorded. Then 10 microliters rabbit muscle lactic dehydrogenase was added to each tube or cuvette and allowed to remain at room temperature for 15 min. The samples and standards were reread at 340 mμ to get the final O.D. A duplicate blank was run with each set of samples and standards. This blank was subtracted from all calculations.

Several researchers have published NAD and NADH values for rat liver. Although the procedures varied, it might be well to report their basic methodology and the values obtained. Originally, the method of Caiger et al. (ref. 28) was employed in this laboratory. This method provided unsatisfactory results because of difficulties in NADH extraction.

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\* Standards were established at concentrations of  $5 \times 10^{-4}$ ,  $5 \times 10^{-5}$ , and  $1 \times 10^{-5}$  M.



Caiger used the method of Jacobsen and Kaplan (ref. 29). NAD was extracted in 0.5 M trichloroacetic acid by using a 1:15 dilution (1 g/15 ml TCA). The enzymatic reaction involved the oxidation of ethanol to acetaldehyde with reduction of NAD to NADH in the presence of alcohol dehydrogenase. Spectrophotometric measurements were made at 340 m $\mu$ .

Similarly, NADH was extracted by using 0.1 M sodium carbonate in a 1:15 dilution in a boiling water bath. After homogenization, the NADH extractant was frozen in liquid nitrogen. This reaction involved the reduction of acetaldehyde to ethanol with simultaneous oxidation of NADH to NAD in the presence of alcohol dehydrogenase. Caiger et al. and Jacobsen and Kaplan decapitated the animals.

Glock and McLean (ref. 30) used a method termed "more sensitive" in the estimation of NAD and NADH with alcohol dehydrogenase, ethanol, and acetaldehyde. The reduction of cytochrome C reductase was followed at 550 m $\mu$ . Extraction of NAD was accomplished by homogenization in 0.1 N hydrogen chloride in a hot water bath. NADH was extracted by homogenization in 0.1 N sodium hydroxide in a hot water bath. Glock and McLean also decapitated the animal.

Bassham et al. (ref. 31) used the extraction method of Glock and McLean but had difficulty with the cytochrome C reductase system. The method of Lowry et al. was used to measure the reduced NAD fluorometrically. Bassham et al. also decapitated the animal.

Lowry et al. (ref. 9) used a somewhat different extraction method coupled with enzymatic cycling and fluorometry to measure oxidized and reduced pyridine nucleotides.

Table A-V-1 shows the NAD and NADH values (in micromoles per gram wet or frozen liver) obtained by these investigators.

## 7. Arterial Blood Lactate and Pyruvate

Lactate in arterial blood was determined by the method of Krasnow (ref. 10) and modified to facilitate further speed and accuracy.

The rat was lightly anesthetized with ether. A medial abdominal incision was made and 4 ml of blood withdrawn from the aortal bifurcation. The blood was added to 8 ml of 10% trichloroacetic acid, mixed and centrifuged for 10 minutes. The buffer was 0.5 M instead of 0.2 M glycine semicarbazide to maintain the pH.

Table A-V-1. Values for rat liver NAD and NADH obtained by different investigators.

<u>Investigator</u>	<u>NAD, micromoles/kg</u>	<u>NADH, micromoles/kg</u>	<u>NAD/NADH Ratio</u>
Authors (Table V)	608	78	8.34
Burch and Von Dippe (ref. 8)	700	50	14.0
Chance and Jamieson (ref. 17)	862	113	7.8
Lowry et al. (ref. 9)	628	252	2.5
Caiger et al. (ref. 28)	424	108	3.9
Bassham et al. (ref. 31)	485	152	3.2
Jacobsen and Kaplan (ref. 29)	446	166	2.7
Glock and McLean (ref. 30)	555	306	1.8

The remaining methodology was the same as that of Krasnow et al. Measurements were made at 366 m $\mu$  with a Beckman DU spectrophotometer.

Pyruvate was determined by another method (Klinische Wochenschrift, 34, 845, 1956). The supernatant (same as that for lactate) was adjusted to pH 7.0 before analysis. Duplicate samples and standards were prepared and analyze at 366 m $\mu$  with a Beckman DU spectrophotometer.

## APPENDIX II

### REPLICATE ANALYSES

#### 1. Q(O/N) and P/O Ratio of Rat Liver Mitochondria

To determine the reproducibility of the Q(O/N) and P/O methods, a Warburg experiment was undertaken by using five 10-min flasks and five zero time flasks. The livers from two 250-g rats were pooled as a homogenate and the mitochondria isolated by centrifugal fractionation. The oxygen consumption and phosphate uptake were measured after 10 min at 30°C, and the base level of phosphate was measured at zero time. The individual values, averages, and standard deviations for oxygen consumption, phosphate uptake, and P/O ratio are presented in Table A-VI-1. The data show that the average P/O ratio of 2.58 falls within the range of 2.2 to 4.1 reported by Copenhagen and Lardy (ref. 12).

Table A-VI-1. Replicate analyses of liver mitochondrial Q(O/N) and P/O from one rat<sup>a</sup>

Flask No.	Oxygen Uptake, μliter/10 min	Oxygen, microatoms	Phosphate Uptake, micromoles	P/O ratio
1	88.0	7.86	20.52	2.62
2	101.7	9.08	23.33	2.57
3	90.1	8.04	20.51	2.55
4	97.9	8.74	23.33	2.67
5	98.3	8.78	21.86	2.49
Avg.	95.2	8.50	21.91	2.58
S.D.	5.8	±0.52	±1.41	±0.07

<sup>a</sup>Substrate, α-ketoglutarate; temperature, 30°C; mitochondrial nitrogen per flask, 1.56 g.

## 2. NAD and NADH in Rat Liver

To test the replication of the NAD and NADH method an experiment was carried out by using only one rat liver. A complete lobe of liver was frozen with Freon-12. Six extractions were made for NAD and six for NADH. In addition, a section of liver tissue was preserved 24 hr at  $-79^{\circ}\text{C}$  (dry ice temperature). After extractions, nucleotides were analyzed by the enzymatic cycling method. The data in Table A-VI-2 indicate the levels of NAD and NADH found. Each extract was assayed in duplicate.

Table A-VI-2. Replicate analyses of liver NAD and NADH from one rat

<u>Extract No.</u>	<u>NAD, micromoles/Kg</u>	<u>NADH, micromoles/Kg</u>
1	705 815	73 79
2	625 595	82 84
3	496 555	77 77
4	700 600	72 73
5	478 480	82 81
6	- -	80 81
Avg.	605	78
7 <sup>a</sup>	495 575	81 82
8 <sup>a</sup>	630 670	94 89

<sup>a</sup>24-hr liver tissue samples.

The most consistent set of replicate values occurred in the NADH assays. This is surprising since NADH can fluctuate considerably under anaerobic conditions. The values obtained for NAD, however, were based upon diluted samples. This could account, in part, for the scattered levels. The effect of storage on frozen tissues does not appear to alter the levels of NAD and causes only a slight increase in NADH.

In general, the average ratio is slightly less than 8.5, which may be due to the large quantity of tissue that was taken for analysis. The freezing of large tissue masses requires additional time; anaerobiosis may occur during this time.

### 3. Lactate and Pyruvate in Rat Arterial Blood

To estimate the variability of the experimental method, lactate and pyruvate were analyzed in six replicate samples from pooled arterial blood of six rats. The results of this analysis are shown in Table A-VI-3. The results indicate that the overall range of values is less than that observed between different animals.

Table A-VI-3. Replicate analyses of lactate and pyruvate in pooled blood from six rats

<u>Samples No.</u>	<u>Lactate, micromoles/ml</u>	<u>Pyruvate micromoles/ml</u>	<u>Lactate/Pyruvate</u>
1	0.499 0.511	0.144 0.132	3.67
2	0.511 0.511	0.126 0.117	4.22
3	0.555 0.519	0.126 0.126	4.25
4	0.499 0.519	0.126 0.135	3.91
5	0.519 0.499	0.139 a	3.66
5	0.499 0.519	0.107 a	4.76
			Avg. 4.08

<sup>a</sup>Data not available because of operational difficulties.

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13. ABSTRACT The use of pure oxygen atmospheres at reduced pressure in manned spacecraft has stimulated new research interest. Exposure of humans to pure oxygen at total pressures of 5 to 7 psia for up to 30 days has generally produced only subjective and occasional symptomatic distress. Nevertheless, studies with animals have clearly documented toxicity if the exposure to pure oxygen is at higher total pressures. The selection of suitable oxygen atmospheres for manned space flight and the development of protective measures against toxic manifestations will be considerably facilitated by a clearer understanding of the toxic syndrome. Rats, dogs and monkeys were exposed to pure (98.5%) oxygen at 1 atm and 1/3 atm in a closed, recirculating chamber and in a nonrecirculating chamber. Rats exposed to a pressure of 1 atm showed significant reduction in efficiency of liver mitochondrial oxidative phosphorylation. Weight loss accompanied this change. Insignificant changes were found in liver or lung mitochondrial oxygen consumption; liver or lung NAD, NADH, or NAD/NADH; and arterial blood lactate and pyruvate, and lactate/pyruvate ratio. A composite index of oxygen toxicity was derived from a multivariant data analysis. Exposure of rats to pure oxygen at 1/3 atm showed slight elevation in P/O ratio in 7 days. The ratio returned to normal in 2 weeks and remained so for 8 months. No significant weight changes were observed. Only preliminary experiments with the dogs and monkeys were conducted, therefore no conclusions can as yet be made. There is an indication that monkeys are more			

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